BRAIN SYSTEMS COORDINATING FEAR TO UNCERTAIN THREATS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2018

Major Subject: Neuroscience

ABSTRACT

Anxiety disorders are among the most common and debilitating forms of mental illnesses in society. Through greater understanding of the fundamental mechanisms of anxiety, as well as of the factors that lead to the persistence and relapse of fear- and anxiety-related symptoms, we may develop novel behavioral and brain techniques for intervention. Emerging evidence in humans and rodent models suggests that the bed nucleus of the stria terminalis (BNST) is a critical brain structure in the regulation and expression of fear and anxious behaviors. However, the precise contributions of the BNST to the expression and relapse of aversive learning and memory are poorly understood. Uncertainty is a key feature in anxiety disorders, and laboratory experiments suggest that the BNST may be required for processing ambiguous signals. Utilizing various modern neuroscientific techniques, including behavioral analyses, intracranial pharmacology, and immunohistochemistry, the current work explored the critical factors and boundary conditions that control BNST-dependent learning and memory. In particular, we utilized an important and clinically relevant animal model—known as Pavlovian fear conditioning, extinction, and relapse—to probe contributions of the BNST to fear- and anxiety-related defensive behaviors. These processes involved exposing rats to pairings of discrete auditory and environmental stimuli (tones and static contexts) with an aversive stimulus (footshock). Animals will come to express conditioned fear responses (defensive immobility) to the conditioned auditory and contextual stimuli alone. These fear behaviors can be extinguished by presenting the conditioned stimuli in the absence of the aversive outcome till fear subsides—relapse of conditioned behaviors can occur after after a variety of aversive triggers. In the current work, we explored the contextual factors that regulate the relapse of extinguished fear, and we

identified the BNST as a critical regulator of relapse, particularly in cases where there is uncertainty of when an aversive stimulus might occur. Temporal uncertainty of an aversive outcome as an overarching factor was tested in detail, revealing a critical role for timing in the recruitment of BNST afferents to learned fears. Primary contributions of the BNST and its neural circuits to conditioned behaviors are analyzed and discussed. In total, this work suggests that temporal and contextual mechanisms, involving the BNST, may contribute to anxious symptoms and relapse. Accordingly, the BNST should be a target of possible therapeutic intervention for anxiety disorders.

DEDICATION

I dedicate this body of work to my parents, David and Joyce, as well as to all of my family and friends for their support and love over the years. Finally, I wish to dedicate this work to anyone who struggles with mental illness.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Stephen Maren, for his guidance, dedication, and wisdom throughout my graduate career. I am extremely grateful to my committee members, Drs. Jim Grau, Mark Packard, and Jun Wang for their wonder insight and support during this journey. I would also like to thank Drs. Naomi Nagaya and Paul Fitzgerald for their invaluable help. I sincerely appreciate the support and contributions from faculty and staff of the Texas A&M Institute for Neuroscience and the Department of Psychological and Brain Sciences at Texas A&M University. I thank Dr. Rachel Smith for generously volunteering her time. I also wish to thank Sylvia Bernal for her help throughout the years.

Broadly, my thanks go to all of my family and friends near and far. I am indebted to all of the students of my graduate program for their friendship and support. I am thankful to all of my current and former lab mates, including Dr. Gillian Acca, Tom Guistino, Janice Kim, Dr. Jingji Jin, Karthik Ramanathan, Reed Ressler, Jocelyn Seemann, Michael Totty, and Dr. Qian Wang. I am extremely grateful to Christine Flood for all her love and encouragement. I also could not be here without the support of my parents, David and Joyce Goode.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Professors Stephen Maren, James Grau, and Mark Packard of the Department of Psychological and Brain Sciences and Assistant Professor Jun Wang of the Department of Neuroscience and Experimental Therapeutics. All work for the dissertation was completed independently by the student, but with generous experimental assistance from members of the Dr. Maren's laboratory.

Graduate study was supported by the Herman F. & Minnie Belle Heep Graduate Fellowship from Texas A&M University (T.D.G.), the Aggies Commit Fellowship from Texas A&M University (T.D.G.), the Close the Gap Fellowship from the National Science Foundation and Texas A&M University (T.D.G.), the Predoctoral Ruth L. Kirschstein National Research Service Award from the National Institute for Mental Health (F31MH107113 to T.D.G.), a grant from the National Institute of Mental Health (R01MH065961 to S.M.), the Memory and Cognitive Disorders Award from the McKnight Foundation (S.M.), and the Brain & Behavior Research Foundation NARSAD Distinguished Investigator Grant (S.M.).

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CHAPTER I

GENERAL INTRODUCTION*

General introduction to the dissertation and specific hypotheses

The primary focus of the present dissertation is to explore the behavioral and brain factors that contribute to the expression and relapse of learned fears, particularly with relation to contributions of the bed nucleus of the stria terminalis (BNST) to these processes. The current dissertation achieves these goals in a series of five chapters; the aims of which are broadly introduced here. Below, we address our aims and predictions for each chapter. To summarize, and in Chapter I, we review and update on our understanding of mechanisms of relapse; these discussions present the reader with common and divergent factors in relapse that are important for subsequent chapters. In Chapter II, we explore mediating behavioral and psychological processes of a trigger for relapse as well as the impact of the test environment on relapse expression. In Chapter III, we begin exploring brain systems supporting relapse in which we examine the BNST's roles in two different forms of relapse. These discoveries build into Chapter IV, in which we test various temporal conditions under BNST inactivation to assess its function in the expression of aversive memories. Finally, in Chapter V, the overarching factors of BNST involvement in fear learning is addressed and discussed. This dissertation includes experiments testing several important and specific hypotheses. Overviews of these hypotheses are discussed here—these predictions are dissected further in each chapter.

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The present Chapter I aims to introduce the reader to important concepts of fear learning, extinction, and relapse that will be essential for all subsequent chapters. In Chapter II, we hypothesized that the exposure of animals to a shock-associated context (rather than shock itself) would be sufficient to induce reinstatement of extinguished fear. Additionally, we tested whether the history of the test context contributed to the expression of relapse in both the short- and long-term. In other words, animals were tested to the extinguished cue in either a context that hosted opposing training paradigms (conditioning and extinction; termed "ambiguous") or just extinction alone ("safe"). In doing so, we determined whether relapse following exposure to the shock-associated ("dangerous") context is mitigated by testing the animals in a context in which only extinction training had occurred. Additionally, we also tested whether the conditioning context itself (rather than a separate context) was sufficient to induce relapse in a safe context. In general, we hypothesized that recent stress (e.g., recent exposure to the dangerous context) would impair extinction recall, and that these outcomes would be promoted in ambiguous contexts, but reduced in safe ones.

In Chapter III, we broadly hypothesized that distinct forms of relapse (e.g., reinstatement vs. renewal; as discussed in Chapter I) may be mediated by dissociable brain regions. In particular, we tested whether the BNST was actively involved in the reinstatement of fear (which depends on recent stress and contextual fear), but not in the renewal of fear (a form of relapse which depends on a mismatch of the extinguished cue with a different, but not necessarily aversive, context). To achieve this goal, rodents underwent conditioning, extinction, and reinstatement or renewal of fear; additionally, rodents were surgically implanted with guided cannulas for local microinfusions of inhibitory drugs into the BNST directly—this allowed us to reversibly inactivate the BNST solely during the expression of relapse.

In Chapter IV, we examined how the timing of an aversive stimulus may affect the contributions of the BNST to learned fears. More specifically, we examined whether the BNST is recruited to fear learning when the cue does not reliably signal the onset of the aversive outcome; independent of the cue's modality or duration *per se*. In other words, we tested whether uncertainty of shock onset (as created during conditioning) regulates the BNST's involvement at fear expression. Temporal uncertainty was achieved using backward conditioning, in which the discrete cue followed—rather than preceded—an aversive shock. This learning was then compared to standard forward (temporally predictable) fear conditioning, in the presence or absence of BNST inactivation. We also studied the extent of activation (as measured by expression levels of the activity-dependent immediate early gene product, Fos) in the BNST and in several of its afferent structures. Our hypothesis was that there would be distinct patterns of activation in the BNST and in its afferents in of fear-regulating regions (e.g., amygdala, hippocampus, and medial prefrontal cortex) following exposure to a temporally predictable or uncertain threat.

In Chapter V, and to conclude the dissertation, we analyze and review datasets that encompass the broad roles of the BNST in aversive learning. We address in detail how timing of aversive events may regulate BNST circuitry. We conclude with a section that summarizes the findings in the dissertation and how these may relate to these broader interpretations of BNST function.

Introduction to animal models of relapse

More than ever, research in animal models is playing a fundamental role in the development of novel therapies for anxiety. Brain imaging in humans and neuroanatomical

studies in animals continue to reveal substantial overlap in the neurobiological systems underlying emotional memories, including conditioned fear memories (Delgado et al. 2008; Herry et al. 2008; Knapska et al. 2012; Kong et al. 2014; Lissek 2012; VanElzakker et al. 2013). Erasing fear memories without disrupting other memory systems remains a heavily coveted end point of behavioral interventions for anxiety disorders (Kindt et al. 2009; Maren 2011; Monfils et al. 2009; Quirk et al. 2010; Schiller et al. 2010). Indeed, behavioral therapies, such as prolonged exposure therapy, typically suppress rather than erase fear memories (Bouton 1988; Maren 2005). The form of learning thought to underlie these therapies—extinction learning—has been found to be rather labile (Bouton 2000; Hermans et al. 2006). As a consequence, fear can readily overpower extinction, and extinguished fear may return under a variety of conditions (Boschen et al. 2009; Bouton 2002; Ji and Maren 2007; Rachman 1979; Rachman 1989; Vervliet et al. 2012). Relapse of extinguished fear poses a considerable challenge to behavioral therapies for fear and anxiety disorders (Boschen et al. 2009; Kindt et al. 2009; Vervliet et al. 2012).

Over the last several decades, Pavlovian fear conditioning has become the gold standard for studying emotional learning and memory in the laboratory (Goswami et al. 2013; LeDoux 2000; Maren 2008; Mineka and Oehlberg 2008; Rasmusson and Charney 1997). Fear conditioning is observed in both humans and animals and is highly amenable to experimental control and investigation. Fear is highly adaptive; it is essential in motivating defensive behavior in the face of threat (Cantor 2009; Ellis 1982; Giske et al. 2013; Öhman and Mineka 2001; Seymour et al. 2004). However, increased conditioned fear is observed in individuals with anxiety disorders, and fear circuits in the brain are thought to mediate and modulate anxiety (Davis 1992; Fanselow and Gale 2003; LeDoux 2012; Zantvoord et al. 2013). In both animals

and humans, conditioned fear results from the repeated pairing of a neutral, yet detectable, conditioned stimulus (CS) with an aversive, biologically significant unconditioned stimulus (US) (Delgado et al. 2006; Gunther et al. 1997; Maren 2001). In rodent models, stimuli such as brief tones or lights often serve as CSs, and mild to moderate footshocks most commonly serve as USs. Although footshocks themselves produce an unconditioned response (i.e., a circa-strike "activity burst," including vocalizations; Fanselow 1994), they ultimately engender a conditioned fear state that is associated with a host of fear responses, including freezing (i.e., immobility). In rodents, freezing is a defensive response to an inescapable threat (Bolles 1970; Fanselow 1994; Nissen 1946; Riess 1945) and is a highly reliable index of conditioned fear to the CS (or context; see below). Conditioned fear responses (CRs) also consist of changes in autonomic reactivity, including the release of stress hormones and endogenous opioids (Antov et al. 2013; Davis 1979; Fanselow and Bolles 1979; Fanselow et al. 1989; Kull et al. 2012; Merz et al. 2013a; Przewłocka 1990; Soeter and Kindt 2011). As will be discussed later, pharmacological interventions for posttraumatic stress disorder (PTSD) and anxiety often target autonomic responding (Bailey et al. 2013; Cain et al. 2012). Fear acquisition in rodents is remarkably similar to that of the general human population (Galatzer-Levy et al. 2013), allowing Pavlovian fear conditioning to serve as a translational model (Milad and Quirk 2012; VanElzakker et al. 2013).

During fear conditioning, animals encode not only an association between the CS and US but also an association between the context in which they occur and the US (i.e., identified as context fear) (Fanselow 1980; Maren et al. 2013). Importantly, conditioned fear to the CS generalizes across contexts, unlike that of extinction (see below). Contexts include the physical environment surrounding the animal (exteroceptive contexts), as well as the animal's internal

states of being (interoceptive contexts) (Maren et al. 2013). In rodent models, exteroceptive contexts are created with distinct cage odors, changes in the texture of the testing platform, and alterations in background lighting and noise. Interoceptive contexts are inherently subjective to the animal and may consist of unconscious components. Interoceptive contexts include (but are not limited to) states of arousal, drug states, states of deprivation, and temporal states (Bouton 1993; Bouton 2002; Bouton et al. 1990; Cunningham 1979; Davidson 1993; Järbe et al. 1981; Richardson et al. 1986; Servatius and Beck 2005). Context fear is elicited by the exteroceptive context in which the aversive US occurs and is particularly strong with unsignaled USs (i.e., contextual fear conditioning) (Fanselow and Bolles 1979; Waddell et al. 2006).

In contrast to conditioning, extinction is a procedure in which the contingency between the CS and US is degraded by presenting the CS alone many times without the aversive footshock. Context fear associated with the US can also be extinguished by placing the subject in the conditioned context in the absence of any aversive US (refer to Maren et al. 2013). As a result, animals learn that the CS (or context) no longer predicts the aversive US (Bouton 2004; Chang et al. 2009; Hermans et al. 2006; Lolordo and Rescorla 1966; Pavlov 1927), thereby reducing conditioned fear. Extinction has been shown to engage distinct neural circuits that act on and interact with the neural circuits involved in conditioning (Courtin et al. 2014; Herry et al. 2010; Maren 2011; Milad et al. 2006b; Myers and Davis 2002; Orsini et al. 2013). Interestingly, these neural circuits are active during the suppression of fear in humans (Delamater and Westbrook 2014; Milad and Quirk 2012; Milad et al. 2006b). As such, fear extinction in rodents has been argued to model exposure therapy in humans (Bouton 1988; Hofmann 2007; Milad and Quirk 2012). Exposure therapy is used to treat a variety of anxiety disorders, including PTSD (Cahill et al. 2006; Foa 2011; McLean and Foa 2011; Motraghi et al. 2014; Powers et al.

2010; Rauch et al. 2012; Rothbaum and Swartz 2002). Certain cues are thought to be more readily associated with the aversive US, and subsequently, these cues may be resistant to extinction under certain conditions (though this has been met with controversy; Mineka and Öhman 2002; Lueken et al. 2011). Overall, fear-relevant cues in humans (e.g., a picture of a spider or snake) have been shown to be difficult to extinguish (McNally 1986; Öhman et al. 1975a; Öhman et al. 1975b).

Although extinction-based therapies such as exposure therapy are effective at suppressing fear, the long-term efficacy of these treatments is challenged by the propensity of extinguished fear to relapse (Bouton 1988; Rachman 1979; Rachman 1989; Rodriguez et al. 1999; Vervliet et al. 2012). Understanding the nature and causes of fear relapse is essential to developing effective therapeutic interventions in patients with anxiety disorders. In this review, we will focus on the behavioral mechanisms involved in relapse of extinguished fear, taking care to translate work in animal models to humans. Additionally, this review will highlight strategies that are known to enhance the retention of extinction.

Extinction retention: a vulnerable process

Pavlov (1927) was the first to note that extinction procedures produce only a temporary loss of conditioned responding. For example, he observed that presenting a novel stimulus after extinction caused a reemergence of the CR (external disinhibition); moreover, the mere passage of time after extinction resulted in a return of the CR (spontaneous recovery). In the context of aversive conditioning, these and other phenomena indicate that extinction procedures do not erase fear memories; rather, they lead to a new memory that inhibits the representation of the US (thereby reducing conditioned responding) (Bouton 1993; Konorski 1967; Maren 2011; Quirk

2002). The reemergence of fear is clearly inimical to the aims of therapy and is unpleasant for the patient (Vervliet et al. 2012; Vervliet et al. 2013); unrelenting and unmanaged anxiety is certainly not without health risks (Baganz and Blakely 2013; Hou and Baldwin 2012; Kemp and Quintana 2013). In the following sections, we review four fundamental fear relapse phenomena: renewal, spontaneous recovery, reacquisition, and reinstatement. Although each phenomenon is discussed in light of animal research, these forms of fear relapse have also been identified in humans (Hermans et al. 2005; Vervliet et al. 2012; Vervliet et al. 2013). In later sections, we will examine how particular stressors may modulate extinguished fear and how stress factors may relate to relapse of fear in general.

Renewal

A fundamental observation concerning extinction is that it is context-specific (Bouton and Bolles 1979; Bouton and Nelson 1994). That is, when an extinguished CS is encountered outside of the extinction context, renewal of conditional responding occurs (Bouton 2004; Bouton and King 1983; Bouton and Ricker 1994; Neumann and Longbottom 2008; Polack et al. 2013; Vervliet et al. 2013). Renewal of fear to an extinguished CS can occur when the CS is presented outside either the exteroceptive or interoceptive context of extinction (Maren et al. 2013; Maren 2014). Renewal of fear is ordinarily strongest when the extinguished CS is presented back in the conditioning context (extinction and conditioning often occur in separate contexts; Maren 2014). Hence, although fear memories readily generalize across contexts, extinction learning is characteristically limited to the context in which the extinction memory was formed (Bouton 2004; Maren 2014; Rosas et al. 2013). Thus, renewal of fear is a major challenge for clinicians. Suppression of fear in a therapist's office may not readily translate to

other environments in the patient's life (Mystkowski et al. 2002; Rodriguez et al. 1999). Further compounding the issue, renewal is not limited to a single change in context; renewal of fear can occur across multiple extinction sessions in several distinct contexts (Bouton et al. 2006). Renewal of fear can also occur when the animal experiences the CS in a novel environment (Neumann and Kitlertsirivatana 2010; Maren 2014) or in a familiar environment where the animal has never experienced the CS (Polack et al. 2013). In both cases, renewal appears to be mediated by an unexpected occurrence of the CS in any given context. Renewal of fear has received considerable attention over the last decade, and several important brain structures have been identified in the regulation of renewal (Lissek et al. 2013; Maren 2011; Maren et al. 2013; Maren 2014; Zelikowsky et al. 2013a). Given the importance of contextual information in fear responding, renewal is thought to interact with other known forms of fear relapse.

Spontaneous recovery

An extinguished response to a CS also returns merely with the passage of time, a phenomenon termed spontaneous recovery (Bouton 1993, Rescorla 1997; Leung and Westbrook 2010; Rescorla 2004; Pavlov 1927). In this case, a change in temporal context (i.e., a form of interoceptive context) has been suggested to account for the return of fear (Bouton 1993). By this view, recent events may be more strongly associated with one another than with temporally distant ones (Bouton 1988; Bouton 1993; Rescorla 2004), suggesting that renewal processes may interact with spontaneous recovery (Bouton 2002; Bouton 2004). Indeed, presentation of a reminder cue of the extinction context prior to testing attenuates relapse of fear in animal models of renewal or spontaneous recovery (Brooks and Bouton 1993; Brooks and Bouton 1994). Clearly spontaneous recovery is a major obstacle in treatment of pathological fear, as routine and

ongoing therapeutic interventions may not be practical or feasible for the patient. If implemented with respect to time, other strategies that are known to reduce or prevent renewal may be useful in curbing spontaneous recovery.

Reacquisition

Another phenomenon that restores conditioned responding is administering additional conditioning trials after extinction (Bouton 2002; Kehoe and Macrae 1997; Napier et al. 1992; Rescorla 2001), a phenomenon known as reacquisition. After extinction, pairing the CS and US once again rapidly restores conditional responding under the majority of circumstances (Bouton 2002). However, in some cases, the reacquisition of fear to an extinguished CS is slow (Bouton 1986). This is particularly true when reacquisition trials occur in a unique extinction context. For this reason, Bouton (2002) has argued that rate of reacquisition may depend upon the presence of contextual cues associated with conditioning or extinction, noting that when cues for extinction are removed (and replaced with cues for the conditioning context), reacquisition of CR is far more rapid. Thus, similar to spontaneous recovery, reacquisition interacts with contextual information and also reflects the context-dependence of extinction memories. Interestingly, intermittent CS-US pairings alongside CS-alone presentations can, if implemented correctly, deepen extinction and weaken the possibility of reacquisition of fear in the wake of the extinction procedure (Bouton 2002; also see Ricker and Bouton 1996). Through this design, postextinction presentation of a CS-US pairing may call on memories of extinction, rather than of conditioning, and thereby may result in a weakening of reacquisition. Even with this paradigm, however, some reacquisition of fear is likely to occur (Bouton 2002).

Reinstatement

Encountering the US in absence of the CS after extinction has been shown to reinstate fear responding to the CS (Bouton and Bolles 1979; Rescorla and Heth 1975; Westbrook et al. 2002). Reinstatement can occur with either a strong or weak US (i.e., a footshock of a smaller amplitude than that utilized in conditioning). Additionally, the reinstating US is often unsignaled (unlike reacquisition), but reinstatement can occur with a signaled US (i.e., presentation of a cue other than the CS with the US; Bouton and Bolles 1979). According to one view, presentation of the US might reinstate fear by serving as a retrieval cue for the conditioning memory. Alternatively, context-US associations might summate with fear to the CS to promote conditional responding (Bouton 1993; Bouton 2002; Bouton and King 1983). Evidence that reinstatement occurs only in the context in which unsignaled USs are delivered is consistent with this view (Bouton 1984; Bouton 1988; Bouton 1993; Bouton and Bolles 1979; Bouton and King 1983). However, Westbrook and colleagues (2002) argue that reinstatement is not always context specific. In this study, Westbrook and colleagues (2002) extinguished two distinct CSs (termed CS1 and CS2) in two separate contexts (conditioning occurred in context A, CS1 was extinguished in context B, and CS2 was extinguished in context C; letters correspond to unique contexts). Later, rats received a footshock (US) reminder in context B (but not C) and were subsequently tested for retention of extinction in a separate neutral context (context D). Rats exhibited more fear to CS1 than to CS2 when tested in D, suggesting that reinstatement is not context specific. This stimulus-specific reinstatement of fear across contexts appears to be independent of renewal of fear (Westbrook et al. 2002; see also Holland 1990). The notion that reinstatement can be observed outside of the context in which the US reminder is presented will

become important for other reinstatement studies described in this manuscript (e.g., Morris et al. 2005a; Morris et al. 2005b).

In some respects, reinstatement may be determined by the mere aversiveness of the US, such that the fear state induced by an unsignaled US reminds the animal of the state of fear at the time of conditioning, thereby facilitating fear responding. As such, other aversive fear-inducing stimuli might yield reinstatement to the CS. Indeed, the concept of reinstatement in recent years has grown to include other "aversive triggers" of fear responding to the CS. For example, presentation of an unextinguished CS (which induces fear) reinstates fear to a different, extinguished CS (Halladay et al. 2012). Moreover, recent exposure to a conditioned context (i.e., a "dangerous" context) has been shown to reinstate fear to an extinguished CS in a separate context (Morris et al. 2005a; Morris et al. 2005b). Low levels of context fear prior to CS presentation in the testing context do not negate this effect. This work by Morris and colleagues (2005a) suggests, in contrast to Bouton (2002), that reinstatement can occur in a context different from the context in which fear was induced (e.g., via exposure to separate dangerous context) (also refer to Westbrook et al. 2002). Conceivably, the mechanisms of these two forms of reinstatement are different insofar as US-induced reinstatement appears to be context dependent, whereas the reinstatement that follows fear induction is not. That said, both footshocks and dangerous contexts are stressful, and stress may therefore play a role in reinstatement of fear (Jacobs and Nadel 1985). Stress clearly confers susceptibility to anxiety disorders (Callaghan et al. 2013; Cohen et al. 2013; Green et al. 2011; Timmermans et al. 2013), though less is known about the role of stress in relapse. Moreover, stress systems in the brain are known to overlap and interact with fear circuitry (Asan et al. 2013; Tye et al. 2011). As such, examining the role that

stress might play in the relapse of fear is of fundamental importance in understanding and preventing relapse.

Stress and fear relapse

Stress is precipitated by a variety of stimuli, can exert a multitude of physiological effects, and may exhibit both facilitatory and inhibitory effects on fear memory depending on the nature of the stressor and the task at hand (Akirav and Maroun 2013; Baratta et al. 2007; Kim and Diamond 2002; Roth et al. 2012; Sanders et al. 2010; Sapolsky 2003; Trammell and Clore 2013; Wideman et al. 2013). Stress can even alter the memory systems involved in solving the task (Goodman et al. 2012; Packard and Goodman 2012). Moreover, the extent to which an animal has control over the stressor ultimately affects the consequence of stress on behavioral performance (Baratta et al. 2007; Christianson et al. 2013; Kubala et al. 2012; Maier et al. 2006). Recently, Hartley and colleagues (2013) demonstrated in humans that inescapable stress impairs extinction, while controllable stress actually reduces spontaneous recovery. As such, understanding the consequences of stress on fear relapse requires an appreciation of the type and time-course of the stress (i.e., physical vs. psychological, acute vs. chronic vs. intermittent, controllable vs. uncontrollable, etc.), as well as its physiological effect on the animal.

Physical stressors are commonly used in rodents (Heinrichs and Koob 2006) and include footshock, restraint stress, forced swim, nutrient deprivation, and loud noise. Certainly, physical stressors are not without psychological implications; physical stressors are merely differentiated from psychological stressors based on the source of the stress. In contrast, psychological stressors do not place physical strain on the animal's body and include predator odor exposure, social isolation, prolonged elevation, dangerous context exposure, and distress vocalizations

(Deschaux et al. 2012; Fleshner et al. 2004; Hu et al. 2014; McNeal et al. 2014; Morris et al. 2005a; Wallace and Rosen 2000). Psychological stressors may also include exposure to novel stimuli and contexts, as well as associative unexpectancy (i.e., an unexpected occurrence of an extinguished CS in a familiar context; Maren 2014). Introduction or elimination of conspecifics can yield psychosocial stress (Huhman 2006). Psychosocial stressors include social defeat, chronic subordinate colony housing, and maternal deprivation (Fraga et al. 2014; Papciak et al. 2013; Uschold-Schmidt et al. 2013). Although all of the aforementioned stressors are specific to rodent models, analogs of these stressors are implemented in human research, particularly psychological and psychosocial stressors (Björkqvist 2001; Campbell and Ehlert 2012; Hartley et al. 2013; Maner et al. 2008; Schultheiss et al. 2005). Worth noting is that stress responding can also be induced pharmacologically or via electrical stimulation of anxiogenic regions of the brain (Kellet and Kokkinidis 2004; Morris et al. 2005b).

Psychological stressors in rodents have garnered considerable attention in recent years, driven in part by the important role played by psychological stress in the psychopathology of human anxiety disorders. Several studies now highlight the potential risk of fear relapse in the wake of psychological stress. Deschaux and colleagues (2012) demonstrated a return of fear to an extinguished CS after a 30-minute exposure of rats to an elevated platform. The relapse of fear in these rats was blocked by chronic administration of the antidepressant, fluoxetine (administration of fluoxetine occurred over 20 consecutive days; refer to Deschaux et al. 2012). As discussed earlier, Morris and colleagues (2005a) induced relapse of extinguished fear in rats simply by briefly exposing the animals to a dangerous context. This effect was blocked with the beta-adrenergic antagonist propranolol (Morris et al. 2005b). Additionally, Morris and colleagues (2005b) demonstrated that artificial induction of adrenergic activity with acute

systemic administration of epinephrine replicated the effects of exposure to the dangerous context. Thus, reinstatement of fear to an extinguished CS may be related to the stress engendered by other unsignaled footshocks or fear in general (Halladay et al. 2012; McCarty and Kopin 1978; Morris et al. 2005b). By this view, any aversive experience might result in the reinstatement of extinguished fear. Whether stress-induced relapse and reinstatement are mediated by overlapping neural structures has yet to be fully explored. If there is overlap, behavioral and pharmacological strategies that curb reinstatement may also attenuate stress-induced relapse. Likewise, stress reduction techniques may be key to reducing vulnerabilities to fear relapse. The effect of physical stress on fear responding in the aftermath of extinction has gone largely unexplored, though a similar pattern is expected as with psychological stressors. Nonassociative mechanisms that accompany stress should also be explored with regards to relapse.

Stress prior to conditioning has been shown to facilitate fear learning in rodents and to make fear responding more resistant to attenuation under certain conditions (Corley et al. 2012; Long and Fanselow 2012; Maren and Chang 2006; Rau et al. 2005; Rau and Fanselow 2009; Rodrigues et al. 2009). Additionally, stress prior to extinction (but after conditioning) can also impair the acquisition of extinction (Adamec et al. 2006; Maren and Chang 2006; Maren 2013). In rodents, the experience of aversive stress prior to conditioning can impair the retention of extinction memories in retrieval tests. For example, single prolonged stress prior to conditioning has been shown to enhance the renewal of fear in extinguished rats (Knox et al. 2012). Additionally, extinguished context fear is poorly retained in rats that have undergone a single prolonged stress procedure prior to the context conditioning procedure (Yamamoto et al. 2008). Similarly, Goswami and colleagues (2010) showed that exposure to predator threat prior

to fear conditioning weakened the acquisition and recall of extinction in a cohort of Lewis rats (described as "PTSD-like"). These PTSD-like rats were shown to exhibit low levels of exploratory behavior on an elevated plus maze. Interestingly, Goswami and colleagues (2010) demonstrated that this impairment in extinction retention was not observed in rats that had previously exhibited high levels of exploratory behavior on the elevated plus maze (i.e., "resilient" rats). In humans, a similar pattern exists in both the facilitation of conditioning and the weakening of extinction through stress (Milad et al. 2008; Peri et al. 2000; Robinson et al. 2013; VanElzakker et al. 2013).

Early life stressors long before fear conditioning may also contribute to the susceptibility of fear relapse. Acute maternal deprivation has been shown to foster a propensity for relapse of fear in young rat pups (Cowan et al. 2013). Interestingly, infant pups under standard rearing conditions exhibit a degree of resistance to relapse; fear fails to reinstate or renew in these rats (but see Revillo et al. 2013). However, the lifelong implications of acute maternal deprivation on fear responding remain unclear. That said, prenatal stress (i.e., stress in pregnant rats) has even been found to reduce the retention of extinction of offspring trained later in life (Green et al. 2011).

Individual differences and susceptibility to relapse

Anxiety disorders exist throughout the world, but certain groups are preferentially affected. For example, women have an increased risk of clinical anxiety and PTSD compared with men (Foa and Street 2001; Kobayashi et al. 2012). Sex differences are observed in animal models of fear conditioning and extinction, as well as in stress responding (Farrell et al. 2013; Gupta et al. 2001; Lebron-Milad et al. 2013; Lynch et al. 2013; Maren et al. 1994; Merz et

al. 2013a; Merz et al. 2013b; Milad et al. 2009). Humans exhibit a similar pattern, albeit with fewer consistencies across studies (Milad et al. 2006a). Nevertheless, it stands to reason that susceptibility of relapse may differ between sexes. Indeed, female rats are more likely to exhibit renewal of fear (Baker-Andresen et al. 2013). Chronic stress prior to conditioning preferentially impairs the recall of extinction memories in male rats, but not female rats; however, unstressed female rats appear to not extinguish as robustly as males (Baran et al. 2009).

The developmental stage of the animal can also have a profound impact on the nature of fear acquisition and expression of extinguished fear (Callaghan and Richardson 2013; Campbell and Ampuero 1985; Kim and Richardson 2007a; Kim and Richardson 2007b; Kim and Richardson 2010; Mactutus et al. 1982; Sanders 2011). Stress responding can vary widely across development in both humans and animals, suggesting that the occurrence of relapse may interact with developmental stages (Green and McCormick 2013; Revillo et al. 2013; Takahashi et al. 1991; Wright et al. 2012). For example, postweanling rats as young as a few weeks old are capable of renewal, spontaneous recovery, and reinstatement, whereas preweanling rats are known to exhibit resistance to relapse of conditioned fear (Kim and Richardson 2010; but see Revillo et al. 2013). Adolescent rats are particularly susceptible to fear relapse when compared with preadolescent and adult rats (Baker et al. 2013). To combat this susceptibility, Baker and colleagues (2013) have shown that a CS-alone presentation (i.e., a retrieval trial) prior to or following extinction training reduced the risk of subsequent renewal in the adolescent rats. Interestingly, Sanders (2011) demonstrated a deficit in renewal of extinguished fear in aged mice (17 months). This impairment in renewal may be related to the weakening of contextual gating systems in the aging mouse brain.

These studies suggest that strategies of relapse prevention should be targeted to susceptible groups accordingly. Drug treatments in humans must certainly be mindful of developmental stages in children and adolescents (Huemer et al. 2010). Furthermore, future work should ascertain how sex differences interact with developmental stages across the lifespan. Several genes are associated with an increased risk for PTSD and other anxiety disorders (Almli et al. 2014; El-Kordi et al. 2013; Erhardt and Spoormaker 2013; Felmingham et al. 2013; Norrholm et al. 2013; Wilker et al. 2013), and these genes may also predispose individuals to relapse. In addition, epigenetic mechanisms of stress and psychopathology have received considerable attention in recent years (Maddox et al. 2013; Norrholm et al. 2013; Zovkic et al. 2013), and these factors might also confer individual differences in fear extinction and relapse. Indeed, some individuals extinguish fear rapidly and exhibit resilience in the face of stress (Franklin et al. 2012; Jovanovic and Ressler 2010; Galatzer-Levy et al. 2013). Insight into what contributes to resiliency, both in terms of behavior and brain function, may offer a means to reduce susceptibility of fear relapse in others.

Strategies to prevent relapse

To this point, we have discussed factors that influence the return of extinguished fear. We will now describe classic and contemporary strategies that may combat relapse of fear.

Optimizing behavioral therapy

Several studies indicate that the magnitude and duration of fear reduction after extinction relates to the amount of extinction training (Cain et al. 2003; Denniston et al. 2003; Orinstein et al. 2010). Extinction learning is slowly acquired, and limited training leads to rapid fear relapse.

Thus, animals undergoing massed extinction trials often experience several hundred consecutive CS-alone presentations (Laborda and Miller 2013; Urcelay et al. 2009). As is often the case in procedures for massed extinction, the intertrial interval may be of short duration, which may in actuality weaken the extinction memory (Li and Westbrook 2008). Spaced trials may offer greater protection from fear relapse than with massed trials (Li and Westbrook 2008; Urcelay et al. 2009). As mentioned earlier, spaced trial procedures across multiple points in time may buffer against spontaneous recovery by supporting multiple temporal contexts with which extinction training is associated (Bouton 2002; Tsao and Craske 2000; Urcelay et al. 2009). The greater efficacy of spaced versus massed extinction trials continues to be explored (Li and Westbrook 2008; also see Fitzgerald et al. 2013).

Extinction in multiple contexts has been argued to facilitate suppression of fear in future encounters with the CS (Balooch et al. 2013; Chelonis et al. 1999; Gunther et al. 1998). This strategy has been argued to be particularly effective at reducing renewal. This has come with mixed results, however (Fitzgerald et al. 2013). Recent experiments suggest that extinction in multiple contexts is not always successful in preventing renewal (Bouton et al. 2006). Pairing massed extinction with extinction in multiple contexts may help foster greater fear suppression than with either strategy alone (Laborda and Miller 2013; Vervliet et al. 2013). Interestingly, by extinguishing renewed fear as a result of an extinguished CS presentation in a novel context, rats exhibit resistance to subsequent renewal of fear when the CS is presented in the conditioning context (Holmes and Westbrook 2013). Although contexts are distinct for experimental animals, several features and cues in the contexts are often shared among different testing chambers and contexts. During times of high risk of relapse, presentation of retrieval (reminder) cues of the extinction experience may foster resilience against renewal and spontaneous recovery or against

relapse in general (Bouton 2000; Brooks and Bouton 1993; Brooks and Bouton 1994; Culver et al. 2011; Dibbets et al. 2008; Dibbets et al. 2013). As a preventative measure, it has been proposed that having patients consciously identify similarities between the extinction context and other environments may prove helpful in preventing relapse (Bouton 2002).

Enriched environments are known to offer protection against the negative effects of stress (Baldini et al. 2013; Mitra and Sapolsky 2009). In turn, supportive and enriched environments may offer protection against stress-induced relapse. Additionally, voluntary exercise has been shown to foster resilience in animals (Fox et al. 2008; Salem et al. 2009; but see Hare et al. 2012). In rodents, voluntary exercise most often comes in the form of wheel running. With more time spent on an exercise wheel, uncontrollable stress exerts less of a negative impact on the animal (Greenwood et al. 2005). Thus, voluntary exercise may help buffer stress-induced fear relapse.

Time of day effects can also play a role in the acquisition and retention of extinction. Recent work in humans highlighted a deepening of extinction simply through training subjects in the morning as opposed to evening sessions (Pace-Schott et al. 2013). In rodent behavioral work, experimenters should be mindful of renewal effects that may occur as a result of irregularity in the time of day at testing. Although patients may be reluctant to entertain the idea, habituation to the US has also been argued to potentially reduce the risk of fear relapse to CS (Rauhut et al. 2001). Other work in humans suggests that merely observing others undergoing extinction can provide some protective effects (Golkar et al. 2013). Interestingly, postexposure sleep appears to enhance long-term fear reduction (Kleim et al. 2013). In another sleep study, Wixted (2013)described a means by which fear can be extinguished during sleep by exposing subjects to odor cues during sleep. In combination with other extinction strategies, these and other novel

procedures may offer greater protection against relapse; however, that remains to be demonstrated.

Behavioral interventions for PTSD in humans are often implemented immediately following trauma (Agorastos et al. 2011; Kearns et al. 2012; Roberts et al. 2009). In recent years, the efficacy of immediate extinction procedures has come under question. In some instances, immediate extinction has been found to be robust and effective in preventing relapse (Myers et al. 2006); there's even some evidence that these procedures are capable of erasing the original fear memory (Maren 2011; Monfils et al. 2009). On the other hand, immediate extinction after conditioning is often ineffective in long-term suppression of fear in rodents (Archbold et al. 2010; Chang and Maren 2009; Maren 2011; Maren 2013; Maren and Chang 2006). In general, delayed extinction has been shown to be far more effective in humans in preventing relapse in renewal and spontaneous recovery paradigms (Huff et al. 2009). Similarly, Archbold and colleagues (2013) found spontaneous recovery in rats to be more pronounced shortly after extinction training (i.e., between 1 and 4 hours postextinction) when compared with later time points (i.e., 8 to 24 hours postextinction). Long-term consolidation of the extinction memories may account for the reduction in spontaneous recovery at the later time points (Archbold et al. 2013). Distributing extinction training across a wide array of time points may deepen extinction (Gershman et al. 2013), and this notion is supported by clinical work in humans (Rowe and Craske 1998; Tsao and Craske 2000).

Pharmacotherapeutic interventions

Pharmacological therapies often coincide with behavioral interventions of anxiety disorders (Choi et al. 2010; Davis et al. 2006; de Kleine et al. 2013; Dunlop et al. 2012; Hetrick

et al. 2010). Several pharmacological strategies have been identified that are known to facilitate extinction learning in animals (for a recent review, see Fitzgerald et al. 2013). In combination with behavioral therapies, these strategies may offer greater protection from relapse (Foa et al. 2002). Recently, a single L-dopa administration was shown to be effective in preventing renewal of fear in rats (Haaker et al. 2013). As mentioned, acute propranolol administration in the wake of a stressful exposure to a dangerous context has been shown to prevent relapse of fear (Morris et al. 2005b). In modulating glucocorticoid signaling, cannabinoids may offer protection in the form of enhanced extinction learning (de Bitencourt et al. 2013; Ganon-Elazar and Akirav 2012; Rabinak and Phan 2013). Systemic administration of the cholinergic antagonist scopolamine has been found to reduce renewal of fear in low doses, perhaps through disruption of both hippocampal activity and normal context-dependent encoding of extinction (Zelikowsky et al. 2013b). D-Cycloserine has also shown some efficacy in reducing anxiety (Bouton et al. 2008), although its effects on extinction are mixed (Fitzgerald et al. 2013). In patients with generalized anxiety disorder, chronic administration of the melatonergic antidepressant, agomelatine, has been found to be efficacious in reducing relapse (Stein et al. 2012). In the future, more uncommon molecular targets might pave the way for future relapse therapies. For example, global increases of magnesium levels in the brain have been shown to improve extinction retention (Abumaria et al. 2011). Pharmacologically enhanced brain-derived neurotrophic factor signaling in the brains of female mice attenuates renewal of fear (Baker-Andresen et al. 2013). In another study in rats, systemic injection of fibroblast growth factor-2 has been shown to also offer protection against renewal (Graham and Richardson 2010).

Deep brain stimulation

Specific regions of the prefrontal cortex (PFC) in rodents and humans have been implicated in fear suppression and resilience (Courtin et al. 2014; Maier et al. 2006), and the PFC has an important role in extinction (Chang and Maren 2011; Herry 2010; Likhtik et al. 2005; Likhtik et al. 2014; Maren 2011; Maroun 2013; Milad et al. 2007; Sotres-Bavon et al. 2012). Stress is thought to perturb these areas of the PFC (Akirav and Maroun 2007; Maier and Watkins 2010; McEwen and Morrison 2013), and one strategy for facilitating extinction involves functional activation of the PFC. By artificially stimulating these regions, resilience in the face of stress might be encouraged, possibly offering a means of relapse prevention. In most cases, artificial stimulation in the brain has been examined only in light of facilitating the extinction process, rather than observing whether its use results in a more resilient form of extinction. That said, others have begun to elucidate the significance of cortical stimulation in relapse prevention. High-frequency stimulation in the PFC of rats has been shown to be effective in preventing reemergence of extinguished fear as a result of a subconditioning procedure (Zheng et al. 2013). Subconditioning involves pairing the US once again with the CS after conditioning; however, the US is dramatically weaker. In turn, the postextinction subconditioning procedure will not initiate reconditioning of fear to the CS but will impair the retention of extinction in future tests. Unlike chronic fluoxetine administration in a separate experiment from the same laboratory, Zheng and colleagues (2013) demonstrated that high-frequency stimulation of the PFC did not buffer against acute stress-induced relapse elicited by exposure to an elevated platform (Deschaux et al. 2012). Cortical stimulation may not be entirely effective alone, but combinative strategies (i.e., behavioral training in conjunction with stimulation) may prove fruitful. Moreover, high-

frequency stimulation is not the only method by which resilience may be produced. In a trace-conditioning paradigm, pairing extinction training with low-frequency stimulation in anterior cingulate cortex (a technique that is known to reduce neural excitability and ultimately induce long-term depression) reduced spontaneous recovery in primates (Klavir et al. 2012). Lastly, there is even evidence that peripheral vagus nerve stimulation coinciding with extinction training can facilitate rapid fear suppression (Peña et al. 2013).

Conclusions

The relapse of fear after exposure therapy is a major challenge for clinical interventions for fear and anxiety (Vervliet et al. 2012). Although we have focused on fear recovery phenomena in isolation of one another, it is important to consider that the conditions that precipitate these phenomena might be concurrently experienced to produce additive or super-additive effects on relapse. For example, experiencing both a passage of time with a change in context after extinction (i.e., a paradigm including both spontaneous recovery and renewal components) might result in a particularly strong relapse of fear (Laborda and Miller 2013). Consistent with these ideas, emerging evidence suggests that relapse phenomena (such as spontaneous recovery or reinstatement, for example) may be mediated by separate neural mechanisms (Ma et al. 2012) and in turn may require alternative modes of intervention. Critically, our understanding of these relapse phenomena has been notably advanced by the study of Pavlovian fear conditioning and extinction in rodents, which provide a unique and unparalleled opportunity for concurrent behavioral and neural analyses. Nonetheless, more work is clearly needed to understand the myriad of factors that cause fear to return in anxious brains.

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CHAPTER II

REINSTATEMENT OF FEAR AFTER EXPOSURE TO A DANGEROUS CONTEXT**

Introduction

Relapse of extinguished fear is common to behavioral therapies for pathological anxiety (Rachman 1979, 1989; Kehoe and Macrae 1997; Boschen et al. 2009; Dibbets et al. 2013; Vervliet et al. 2013a,b; Bouton 2014; Haaker et al. 2014). This is a pervasive issue: Craske and Mystkowski (2006) suggest that as many as three out of five patients will experience significant relapse of previously extinguished fear. Fortunately, fear reduction and relapse phenomena in humans can be effectively modeled using Pavlovian conditioning and extinction procedures in rats (Bouton 1988; Delgado et al. 2006; Milad et al. 2006; Hofmann 2007; Maren 2011; Milad and Quirk 2012; VanElzakker et al. 2013). Fear conditioning involves the coupling of a neutral, yet detectable, conditioned stimulus ("CS"; e.g., an auditory tone) with a potent, biologically significant unconditioned stimulus ("US"; e.g., unavoidable footshock) (Pavlov 1927; Gunther et al. 1997; Maren 2001, 2005). After conditioning, the CS comes to elicit conditioned fear responses ("CRs"), such as freezing behavior in rats (Bolles 1970; Fanselow 1980, 1994; Sigmundi et al. 1980; Hagenaars et al. 2014).

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After repeated presentations of the CS alone, the magnitude and frequency of the CR is diminished, a process termed extinction (Pavlov 1927; Konorski 1948; Lolordo and Rescorla 1966; Rescorla 2001; Bouton 2004; Hermans et al. 2006; Chang et al. 2009; Fitzgerald et al. 2014; also see Jones et al. 2013). Standard extinction procedures do not erase the original fear memory; rather extinction represents a new form of learning that inhibits conditioned responding to the aversive CS (Konorski 1967; Bouton 1993; Falls 1998; Maren 2011; also see Myers et al. 2006). Consequently, extinguished fear in humans and other animals is often transient and prone to different forms of fear relapse, including "renewal," "spontaneous recovery," and "reinstatement" (Bouton 2000, 2002, 2014; Ji and Maren 2007; Schiller et al. 2008; Maren 2011; Goode and Maren 2014). Presentation of an extinguished CS outside of the context in which extinction training occurred—whether in a novel or familiar place—can induce "renewal" of fear to the CS (Bouton and King 1983; Bouton and Ricker 1994; Alvarez et al. 2007; Effting and Kindt 2007; Neumann and Longbottom 2008; Polack et al. 2013; Vervliet et al. 2013a; Maren 2014). Fear renewal can also occur if the extinguished CS is encountered during a time in which the animal's interoceptive context is incongruent with the internal state that is associated with extinction (Bouton 1993; Bouton et al. 2006; Maren et al. 2013; Vervliet et al. 2013a,b). For recent reviews on the function of contexts in conditioned and extinguished fear, see Maren et al. (2013) and Urcelay and Miller (2014). With relation to renewal of fear (see Bouton 2002), "spontaneous recovery" is a return of extinguished CR that occurs with the mere passage of time (Pavlov 1927; Baum 1988; Quirk 2002; Rescorla 2004). Finally, reexposure to the US alone after extinction can result in a return of fear responding to the CS, termed "reinstatement" (Pavlov 1927; Rescorla and Heth 1975; Bouton and Bolles 1979; Bouton 1988, 1991; Westbrook et al. 2002; Hermans et al. 2005; Norrholm et al. 2006; Haaker et al.

2014; also see Dirikx et al. 2009). Recent reports indicate that exposure of rats to cues or contexts that have been independently associated with an aversive US can induce reinstatement. For example, Halladay et al. (2012) have reported that presentation of a nonextinguished CS will reinstate fear to an extinguished CS. Similarly, Morris et al. (2005a,b) found that brief exposure of rats to a shock-associated context—minutes before presenting an extinguished CS in a separate testing context—reinstated fear to the CS. This fear enhancing effect persisted at least 24 h following the "dangerous" context exposure. These findings have important implications for fear relapse after extinction-like therapies in humans. That is, they suggest that reinstatement can occur not only after an aversive event, but also after exposure of individuals to contexts or cues associated with aversive experiences in the past.

Of course, one factor that is known to influence relapse is the context in which the extinguished CS is experienced. For example, Morris et al. (2005a,b) conditioned, extinguished, and tested rats to an auditory CS in the same context (refer to Experiments 3, 5, 6, and 7 of Morris et al. 2005a, and Experiment 1 in Morris et al. 2005b). As a result, the test context was "ambiguous" because it had hosted two distinct training experiences: an aversive conditioning episode and a "safe" extinction episode (see Bouton 1988, 2002). Because contextual information is thought to "set the occasion" for the current meaning of the CS (Holland 1985; Bouton and Swartzentruber 1986; Bouton 1993, 1997; Miller and Escobar 2002), relapse of fear may be more likely in a context that has previously hosted conditioning. By this view, relapse might be thwarted by testing rats in a reliably safe context (e.g., a context that has hosted only extinction training). Consistent with this, Bouton and Swartzentruber (1989) demonstrated that reacquisition of extinguished CR (i.e., a return of CR after pairings of the extinguished CS with the US) was slower in a context that solely hosted extinction training when compared with

reacquisition in a novel context or with reacquisition in the original conditioning context (refer to Experiment 2 of Bouton and Swartzentruber 1989; also see Leung et al. 2007). Ultimately, contexts with a history of hosting both conditioning and extinction may interfere with the animal's ability to discriminate between these training episodes (see Bouton and Bolles 1979), thus fostering relapse.

Therefore, in the present experiments, we examined whether the associative history of the test context influences the expression of fear relapse to an extinguished CS in rats. Specifically, we hypothesized that reinstatement of fear would occur in an ambiguous test context that had previously hosted both conditioning and extinction, but not in a "safe" test context that had only predicted the absence of shock (e.g., an extinction-only context). To address these predictions, we first established a "dangerous" shock-associated context (or a "neutral" no-threat context) prior to conditioning and extinction. After extinction, we exposed rats to either the dangerous context (i.e., the relapse trigger) or the neutral context prior to retrieval testing in either an ambiguous or safe context. Retrieval tests were conducted at 30 min ("short-term") and/or 24 h ("long-term") after exposure to the dangerous or neutral context. Morris et al. (2005a) reported that relapse was most robust soon after exposure to a dangerous context; we were particularly interested to determine whether testing in a safe context would mitigate relapse soon after exposure to the dangerous context. We also examined whether brief exposure of rats to the conditioning context—in place of the unsignaled shock context—would induce relapse in the safe test context. Overall, our work indicates that relapse of extinguished fear interacts with both the associative history of the testing context and the recency of exposure to the dangerous context. Specifically, short-term relapse occurred in both test contexts following exposure of rats to the unsignaled shock context, whereas long-term relapse occurred only in the ambiguous

context. We observed no relapse of fear in the safe context following exposure of rats to the conditioning context. This work demonstrates the susceptibilities of extinction memories to disruption in the wake of psychological stress, and highlights the importance of context associations in modulating fear responding.

Results

Experiment 1a/b: fear relapse in an ambiguous context after exposure to a dangerous context

We first sought to replicate the findings of Morris et al. (2005a) by determining whether exposure to a dangerous context would cause fear relapse to an extinguished CS. Therefore, in Experiment 1, we examined whether fear relapse would occur in an ambiguous retrieval context at short- and long-term intervals following exposure of rats to a separate dangerous context (refer to Table 1 for an overview of the experimental design). Data from the extinction and testing sessions are shown in Figure 1. No significant group differences were detected for fear conditioning in any of the following experiments (conditioning data not shown). Mean freezing (±SEM) prior to CS onset for the first extinction session is as shown: DANGER/EXT = 83.6% ± 3.5%, NEUTRAL/EXT = 60.2% ± 7.1%, DANGER/NoEXT = 68.2% ± 4.9%, NEUTRAL/NoEXT = 64.6% ± 5.9%. Baseline freezing revealed a main effect of exposure assignments [$F_{(1,60)} = 6.02$; $P < 0.05$]. No other group differences were detected for baseline fear for the first extinction session. Overall, rats exhibited robust extinction and significantly decreased their levels of fear by the final block of extinction training (see Fig. 1A, under "Extinction"). This impression was confirmed in a repeated-measures analysis of variance (ANOVA) that revealed a significant main effect of block [$F_{(1,60)} = 214$; $P < 0.0001$]. Extinguished rats (EXT) exhibited higher fear after CS onset compared with NoEXT rats

(significant block × extinction interaction [$F_{(1,60)} = 19.0$; $P < 0.0001$]). Interestingly, DANGER rats (i.e., DANGER/EXT and DANGER/NoEXT) were slower to extinguish their freezing behavior. Specifically, we observed a block × exposure interaction [$F_{(1,60)} = 6.88$; $P < 0.05$], indicating that DANGER rats were freezing more in the first block of extinction training as compared with NEUTRAL rats. Of course, rats in the DANGER groups received one more footshock than those in the neutral groups, and this may have retarded extinction (context fear at baseline may have bolstered CS responding in DANGER rats). Nonetheless, the exposure × extinction interaction was not reliable across the entirety of extinction training [$F < 1.00$] and no significant differences in the groups were detected for the final block of extinction training.

	Phase					
EXP. #	Establish DANGER or NEUTRAL Context	Condition	Extinction (EXT or NoEXT)	Exposure	Short-term test	Long-term test
AMB						
1a)	A+ or A−	BT+	BT− or B−	A−	BT−	BT−
1b)	A+ or A−	BT+	BT− or B−	A−	n.a.	BT−
SAFE						
2a)	A+ or A−	BT+	CT− or C−	A−	CT−	CT−
2b)	A+ or A−	BT+	CT− or C−	A−	n.a.	CT−
3)	n.a.	BT+	CT− or C−	B− or C−	CT−	CT−

Experimental designs are read from left to right, with numbers corresponding to Experiments 1–3.
(A,B,C) experimental contexts, (T) tone CS, (+) US, (−) no US, (n.a.) not applicable, (SAFE) safe testing context, (AMB) ambiguous testing context.

Table 1. Experimental designs.

Twenty-four hours after extinction training, rats were exposed to either a shock-associated context (DANGER) or to a familiar no-threat context (NEUTRAL) for 3 min (reported in Fig. 1A, under "Exposure"). As expected, the dangerous context itself reliably induced fear. Rats in the dangerous context exhibited significantly higher levels of freezing than rats in the neutral context. This was confirmed in an ANOVA that revealed a significant main effect of exposure [$F_{(1,120)} = 145$; $P < 0.0001$] and a significant exposure × minute interaction [$F_{(1,120)} = 6.30$; $P < 0.005$]. Freezing levels in the exposure chambers did not interact with extinction assignments [$F < 1.50$].

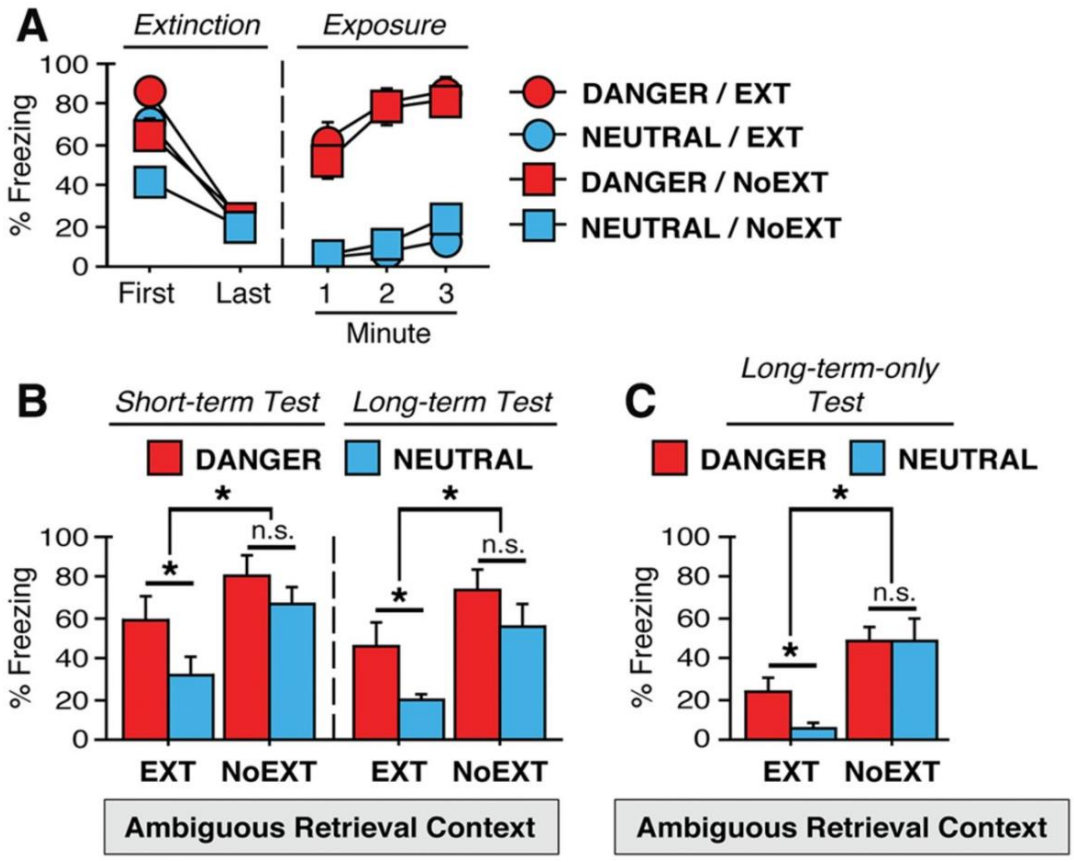

Figure 1. Relapse of extinguished fear in an ambiguous retrieval context at 30 min and 24 h after exposure of rats to a dangerous context. (A) Extinction = mean (±SEM) percentage of freezing during the first 15 post-CS intervals (for "EXT" rats; or equivalent for "NoEXT" rats) on the first day of extinction training ("First"), and during the final 15 post-CS intervals on the last day of extinction training ("Last"). Exposure = mean (±SEM) percentage of freezing during each minute in the shock-associated context ("DANGER") or no-threat ("NEUTRAL") context prior to CS testing. **(B)** Short-term test and Long-term test = mean (±SEM) percentage of freezing during five post-CS intervals (per test) in the ambiguous retrieval context. Rats were tested to the CS in the ambiguous context at 30 min ("Short-term test") and 24 h ("Long-term test") after the exposure phase (Experiment 1a). **(C)** Long-term-only test = mean (±SEM) percentage of freezing during five post-CS intervals in the ambiguous retrieval context. Rats were tested to the CS at 24 h postexposure without short-term testing (Experiment 1b). Asterisks indicate significant differences ($P < 0.05$) for each retention test; (n.s.) nonsignificant comparisons**.**

After exposure to the dangerous or neutral contexts, rats in Experiment 1a were tested to the CS in an ambiguous retrieval context at 30 min and 24 h after the exposure session (refer to Table 1). As shown in Figure 1B, rats exposed to the "dangerous" context exhibited relapse of extinguished fear, relative to nonextinguished animals or animals exposed to a "neutral" context.

An ANOVA revealed a significant exposure × extinction × trial interaction [$F_{(6,168)} = 2.25$; $P < 0.05$] and post hoc comparisons ($P < 0.05$) indicated that DANGER/EXT rats exhibited significantly more freezing across trials and days as compared with NEUTRAL/EXT rats. Conversely, DANGER/NoEXT rats were not significantly different from NEUTRAL/NoEXT rats. The test day × exposure × extinction interaction was not reliable [$F < 0.50$], indicating that relapse of fear in DANGER/EXT rats was apparent for both test sessions. A main effect of test day [$F_{(1,168)} = 6.12$; $P < 0.05$] showed that responding was higher overall for all groups in the short-term test as compared with long-term testing (some extinction of fear is expected over the course of short-term testing). Overall, DANGER rats displayed significantly more fear at both tests compared with NEUTRAL rats (main effect of exposure [$F_{(1,168)} = 7.18$; $P < 0.05$]) and nonextinguished controls exhibited more fear at both tests overall as compared with extinguished rats (main effect of extinction [$F_{(1,168)} = 12.30$; $P < 0.005$]). Baseline context fear prior to CS onset was low (<30%) for all groups across both tests (data not shown). Overall, as predicted, data for Experiment 1a suggest that exposure to the dangerous context caused both short- and long-term reinstatement of fear to an extinguished CS.

In Experiment 1b, the procedures were identical to those in Experiment 1a except we omitted the short-term test. This was done to determine whether short-term testing (which is itself an extinction test) might undermine long-term relapse. However, as shown in Figure 1C, the test data mirrored the results from Experiment 1a. Extinguished rats that were exposed to the dangerous context exhibited significantly more fear to the CS than neutral-exposed rats in the long-term test. A repeated-measures ANOVA revealed a significant main effect of exposure for extinguished rats [$F_{(1,84)} = 8.01$; $P < 0.05$] across all trials, and a significant exposure × trial interaction [$F_{(1,84)} = 2.22$; $P < 0.05$]. In contrast, no main effect of exposure was revealed for

nonextinguished rats [$F < 0.01$], nor did we find any exposure × trial interaction for nonextinguished rats [$F < 0.50$]. Mean baseline context fear (prior to CS onset) for each group was low in the long-term test (<20% freezing; data not shown). In sum, as expected for Experiment 1, we observed relapse of fear in the ambiguous testing context at 30 min and 24 h following exposure of extinguished rats to a dangerous shock-associated context.

Experiment 2a/b: no long-term fear relapse in a safe context after exposure to a dangerous context

The primary objective of Experiment 2 was to determine whether testing rats in a safe (extinction-only) context would blunt fear relapse (refer to Table 1). Mean baseline freezing prior to the first extinction trial is as follows: DANGER/EXT = 45.8% (4.4%), NEUTRAL/EXT = 33.8% (7.1%), DANGER/NoEXT = 59.4% (6.1%), NEUTRAL/NoEXT = 31.6% (5.8%). As in Experiment 1, we observed a main effect of exposure assignments during this baseline [$F_{(1,60)} = 6.46$; $P < 0.05$]; no other significant differences were detected for baseline context fear. Extinction training resulted in a robust suppression of fear (Fig. 2A). This was confirmed by a significant main effect of block in the ANOVA [$F_{(1,60)} = 85.8$; $P < 0.0001$]). EXT rats showed significantly more fear to CS-only presentations compared with the mere exposure of nonextinguished rats to the context (main effect of extinction assignment [$F_{(1,60)} = 24.0$; $P < 0.0001$]). Consistent with Experiment 1, DANGER rats did not extinguish as rapidly as NEUTRAL rats. A significant exposure × extinction × block interaction [$F_{(1,60)} = 10.0$; $P < 0.0001$] indicated that freezing was higher in the first block of extinction training for DANGER rats. Post hoc comparisons ($P < 0.05$) revealed that NEUTRAL/NoEXT exhibited significantly less fear compared with all other groups (i.e., DANGER/EXT, DANGER/NoEXT,

NEUTRAL/EXT) in the first block. There were no significant differences between the groups in the final extinction block (all group means were <40% freezing by the end of extinction).

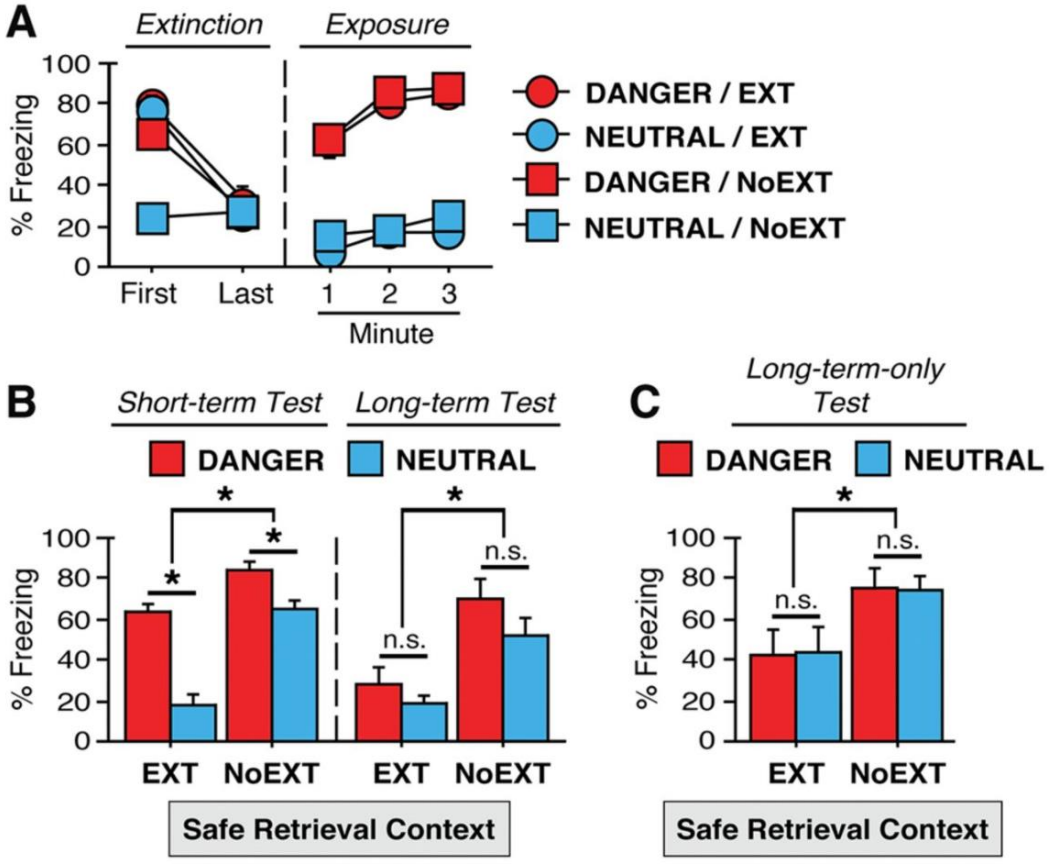

Figure 2. Relapse of fear in a safe retrieval context shortly after exposure to a dangerous context, but no long-term relapse of fear in a safe context. (A) Extinction = mean (±SEM) percentage of freezing during the first 15 post-CS intervals (for "EXT" rats; or equivalent for "NoEXT" rats) on the first day of extinction training ("First"), and during the final 15 post-CS intervals on the last day of extinction training ("Last"). Exposure = mean (±SEM) percentage of freezing during each minute in the shock-associated context ("DANGER") or no-threat ("NEUTRAL") context prior to CS testing. **(B)** Short-term test and Long-term test = mean (±SEM) percentage of freezing during five post-CS intervals (per test) in the safe retrieval context. Rats were tested to the CS in the safe context at 30 min ("Short-term test") and 24 h ("Long-term test") after the exposure phase (Experiment 2a). **(C)** Long-term-only test = mean (±SEM) percentage of freezing during five post-CS intervals in the safe retrieval context. Rats were tested to the CS at 24 h postexposure without short-term testing (Experiment 2b). Asterisks indicate significant differences ($P < 0.05$) for each retention test; (n.s.) nonsignificant comparisons.

Twenty-four hours after extinction training, all rats were exposed to either the dangerous or neutral contexts for 3 min (see Fig. 2A, under "Exposure"). As in Experiment 1, DANGER

rats exhibited significantly more fear in the dangerous context as compared with NEUTRAL rats in the neutral context for Experiment 2. This impression was confirmed in an ANOVA that revealed a significant main effect of exposure [$F_{(1,120)} = 252$; $P < 0.0001$] and a significant minute × exposure interaction [$F_{(2,120)} = 4.49$; $P < 0.05$]; these effects did not interact with extinction history [$F < 1.70$], Overall, the dangerous context reliably induced fear whereas the neutral context did not.

For rats in Experiment 2a, subjects received a retention test in the safe extinction context at 30 min and 24 h following the exposure phase (Fig. 2B). Similar to Experiment 1a, rats exposed to the "dangerous" context prior to the retrieval test exhibited fear relapse to the extinguished CS, despite the fact that testing occurred in the extinction context (i.e., a safe context). However, unlike Experiment 1, testing in the safe context mitigated relapse of fear during the long-term test. These impressions were confirmed in an ANOVA that revealed a significant exposure × extinction × day interaction [$F_{(1,168)} = 5.18$; $P < 0.05$]. Post hoc comparisons ($P < 0.005$) indicated that DANGER/EXT rats exhibited significantly more fear in the short-term test—but not the long-term test—as compared with NEUTRAL/EXT rats. Similar to Experiment 1a, responding was higher across groups in the short-term test as compared with long-term testing (main effect of test day [$F_{(1,168)} = 15.2$; $P < 0.001$]), and freezing was higher in NoEXT rats across trials when compared with EXT rats (main effect of extinction [$F_{(1,168)} = 49.1$; $P < 0.0001$]). Additionally, DANGER rats showed greater levels of fear overall as compared with NEUTRAL rats (main effect of exposure [$F_{(1,168)} = 18.3$; $P < 0.0005$]). Unlike Experiment 1a, DANGER/NoEXT rats exhibited significantly more fear in the short-term test as compared with NEUTRAL/NoEXT rats ($P < 0.05$). These results may reflect the pattern we observed for DANGER/NoEXT rats in the early phases of extinction to the CS, and may be a

unique feature of testing in the safe context (NoEXT rats did not differ across exposure assignments in any of the long-term tests of this report). Overall, the ANOVA on the test data in Experiment 2a suggests that a general increase in fear in rats in the DANGER condition cannot account for the later relapse of fear in extinguished animals. Baseline context fear for each group was low (<30%) prior to CS onset in each test. To summarize, testing in a safe context did not prevent short-term relapse of extinguished fear, but did mitigate the long-term reinstatement of fear.

Additionally, this outcome was confirmed in Experiment 2b, in which rats were submitted to identical procedures except that the short-term test was omitted. As shown in Figure 2C, there was no relapse of extinguished fear in the safe retrieval context 24 h after exposure of rats to the DANGER context. There was only a main effect of extinction [$F_{(1,168)} = 8.96$; $P < 0.01$] in the ANOVA, indicating that nonextinguished rats exhibited significantly more fear at test than extinguished subjects. In sum, testing in a safe context prevented the long-term relapse of fear.

Experiment 3: no relapse of fear in a safe test context following exposure to the conditioning context

In Experiments 1 and 2, we utilized a separate shock context to serve as the dangerous context for the exposure phase prior to CS testing. However, the conditioning procedure itself yields a dangerous context (the conditioning context). Thus, the goal of Experiment 3 was to examine whether brief exposure to the conditioning context could induce relapse (see Table 1). Based on the results we obtained in Experiment 2, we expected the potential for relapse to be weak in Experiment 3 (at least in the long-term test). For the exposures in Experiment 3, we

exposed rats either to the conditioning chamber (DANGER) or to the extinction chamber (SAFE) 30 min prior to a retention test back in the extinction chamber (long-term testing occurred 24 h later).

Figure 3. No relapse of fear in a safe retrieval context following exposure of rats to the conditioning context. (A) Extinction = mean (±SEM) percentage of freezing during the first 15 post-CS intervals (for "EXT" rats; or equivalent for "NoEXT" rats) on the first day of extinction training ("First"), and during the final 15 post-CS intervals on the last day of extinction training ("Last"). Exposure = mean (±SEM) percentage of freezing during each minute of the exposure to the conditioning context ("DANGER") or extinction context ("SAFE") prior to CS testing. **(B)** Short-term test and Long-term test = mean (±SEM) percentage of freezing during five post-CS intervals (per test) in the safe retrieval context. All rats were tested to the CS in the safe context 30 min ("Short-term test") and 24 h ("Long-term test") after the exposure phase. Asterisks indicate significant differences ($P < 0.05$) for each retention test; (n.s.) nonsignificant comparisons.

For Experiment 3, baseline freezing prior to the first extinction trial was not different among the groups (a trending but nonsignificant main effect of exposure assignments was

observed [$F < 4.00$]): DANGER/EXT = 30.7% (10.0%), SAFE/EXT = 48.2% (10.7%), DANGER/NoEXT = 18.5% (5.6%), SAFE/NoEXT = 37.4% (9.4%). As shown in Figure 3A, extinction resulted in a suppression of freezing behavior. As expected, we observed a main effect of extinction assignment [$F_{(1,28)} = 16.5, P < 0.0005$] and a significant block × extinction interaction [$F_{(1,28)} = 13.7, P < 0.001$] such that EXT rats exhibited significantly higher levels of freezing in the first block of extinction training. We observed a main effect of exposure [$F_{(1,28)} = 4.99, P < 0.05$], however this effect was carried primarily by SAFE rats (and not DANGER rats; unlike Experiments 1 and 2) in the first block of the extinction phase. Groups did not significantly differ by the final block of the analyses (group means were <30% freezing by the final block of extinction), so we proceeded to the next phase of behavioral training.

Twenty-four hours after extinction, rats were exposed to either the conditioning context (DANGER) or the extinction context (SAFE) for 3 min (see Fig. 3A, under "Exposure"). As expected, DANGER rats exhibited significantly more fear in the conditioning context as compared with SAFE rats in the extinction context (main effect of exposure [$F_{(1,56)} = 35.0; P < 0.0001$]). Extinction history did not interact with this effect [$F < 0.50$]. A main effect of trial indicated that rats increased in freezing along the course of the exposure session [$F_{(2,56)} = 5.32; P < 0.01$]. No other significant differences were detected for the exposure phase. Overall, the conditioning chamber reliably induced freezing in subjects.

Thirty minutes and 24 h following the exposure session, all rats were tested to the CS in the "safe" extinction context. Despite high levels of fear in the conditioning context, no relapse of fear was observed for either test in the safe context (Fig. 3B). In other words, in contrast to Experiment 2a, we did not observe short-term relapse of fear in the safe context. Specifically, the ANOVA did not reveal a reliable exposure × extinction × day interaction [$F < 0.50$], nor was

there a significant trial × exposure × extinction interaction [$F < 1.00$] across the two test sessions. A main effect of extinction [$F_{(1,168)} = 12.1$; $P < 0.005$] showed that nonextinguished rats froze more at test than extinguished rats, which was expected. A main effect of test day [$F_{(1,168)} = 4.44$; $P < 0.05$] indicated that responding was greater overall on the first day of testing, but some extinction of fear to the CS is expected across test sessions. Group means at baseline were <30% freezing per test in Experiment 3. Collectively, these analyses indicate that although exposure to the conditioning context generated fear, it did not drive relapse of fear at either time point in the safe test context. In sum, Experiment 3 indicates that fear relapse is completely mitigated in a safe test context when exposure to the conditioning context serves as the aversive trigger.

Discussion

In the present study, we have examined contextual factors regulating the relapse of previously extinguished fear. In agreement with Morris et al. (2005a), we have shown that brief exposure of rats to an unsignaled shock-associated context (i.e., a dangerous context) promotes fear relapse to an extinguished CS. Importantly, we have extended on these results by showing that the associative history of the retrieval context influences fear relapse: long-term fear relapse was attenuated in a safe (extinction-only) retrieval context, but not in an ambiguous retrieval context (i.e., an extinction context that had previously hosted conditioning). The safe retrieval context did not prevent relapse altogether, insofar as short-term relapse occurred regardless of where the extinguished CS was tested. Moreover, brief exposure of rats to the conditioning context did not result in relapse of fear in any of the retrieval tests in the safe context. Our findings provide new insights into the factors regulating reinstatement of fear after exposure of animals to aversive stimuli. In particular, strategies aimed at preventing relapse of fear may need

to consider the context in which fearful stimuli are likely to be encountered after therapy. Indeed, safe contexts appear to promote the retention of extinction relative to contexts with a history of both aversive and safe experiences.

Previous research indicates that reinstatement of fear to a discrete CS is driven by context–US associations established in the reinstatement context (Bouton and Bolles 1979; Bouton and King 1983; Bouton 1984; Wilson et al. 1995; Frohardt et al. 2000). In these studies, reinstatement is context-dependent; it only occurs in the context in which the US is delivered. In contrast to these findings, results from our work and Morris et al. (2005a,b) indicate that reinstatement is not always context-dependent. For example, in the present experiments, short-term reinstatement of conditioned freezing to the CS occurred in a context that was never associated with shock (the safe extinction context). Reinstatement under these conditions may be due to mediated conditioning; the CS may have retrieved a context–US association that promoted fear (Holland 1990; Westbrook et al. 2002). However, our current work also indicates that long-term reinstatement of fear (after exposure to a dangerous place) is susceptible to contextual control; it only occurred in a context in which the US had been experienced. In this case, the conditioning context may have encouraged recall of the CS–US memory encoded during the conditioning phase, whereas the extinction context encouraged recall of the CS–"no US" memory (Bouton 1993, 2002; Vansteenegen et al. 2006; also see Bouton et al. 2004). Nevertheless, retrieval cues for extinction training are not always sufficient to prevent relapse (e.g., in short-term reinstatement). For example, previous work has shown that retrieval cues for extinction do not always suppress fear renewal (Dibbets et al. 2008, 2013).

An alternative explanation for the reinstatement of extinguished fear in our study is that it may reflect renewal brought about by a shift in the interoceptive context in the wake of exposure

to a dangerous place (Bouton et al. 2006). That is, extinction training reduces levels of stress and fear; this low-fear state may become an important interoceptive context that helps regulate the expression of the extinction memory. If rats are returned to the extinction context in a state of high stress following fear induction (such as after exposure to a dangerous context), then the animal may experience the extinguished CS outside of the "safe" interoceptive context associated with extinction training. Consistent with this idea, a reduction in physiological arousal (via systemic administration of the β-adrenoceptor antagonist, propranolol) has been shown to prevent relapse following exposure to a dangerous context (Morris et al. 2005b). A shift in interoceptive context might also explain long-term reinstatement insofar as exposure to the dangerous context—at least the unsignaled shock context—may produce a long-lasting stress response. Relatedly, it is conceivable that exposure to the dangerous context either strengthens the fear memory (a form of reconsolidation) or impairs the retention of the extinction memory (Izquierdo et al. 2006; Miracle et al. 2006; Holmes and Wellman 2009; Knox et al. 2012; Deschaux et al. 2013; Hamacher-Dang et al. 2013; Raio et al. 2014; also see Siette et al. 2014). Both of these effects would promote expression of the fear memory, although neither of these explanations allow for the context-dependent expression of reinstatement during the long-term test. On a final note, relapse of fear may also depend on the robustness of the reinstating trigger. For example, we did not observe relapse of fear following exposure of rats to the conditioning context, however this context appeared to induce less freezing behavior when compared with the exposure of rats to the unsignaled shock contexts of Experiments 1 and 2. The safe context may have been able to mitigate relapse in Experiment 3 because rats were not as stressed as in Experiments 1 and 2. Rats did not receive any additional footshocks beyond conditioning in Experiment 3, which may factor in to the expression of relapse.

In conclusion, the present results reveal that mere exposure to a dangerous context promotes the reinstatement of conditioned fear to an extinguished CS. This effect was context-dependent, at least with respect to time: long-term reinstatement only occurred in test context that had previously been associated with shock and was minimal in an extinction context in which shock had never been delivered. These results suggest that the associative history of the retrieval context is an important determinant of reinstatement of extinguished fear.

Materials and methods

Subjects

Subjects were 160 male Long Evans (Blue Spruce) rats from Harlan Laboratories (Houston, TX, USA). $N = 32$ at each test (Experiments 1a, 1b, 2a, 2b, and 3), with an equal number of subjects per group for all phases of training. All rats were 8 wk of age and weighed 200–250 g upon arrival at the vivarium. All rats were individually housed in clear plastic cages on a rotating cage rack (Animal Care Systems, Inc.). Experimental group assignments were randomized for homecage position in the vivarium. Rats were given free access to water and standard rat chow; sawdust served as cage bedding. Clean homecages were provided for the rats once a week, with behavioral testing occurring on separate days from the cage changings. Rats were kept on a fixed light–dark schedule (14 h of light and 10 h of darkness per day; lights on at 7:00 a.m. each day) with all handling and behavioral testing occurring during the illuminated period for the rats. Experimenters handled rats for 1 min a day for 5 d prior to the start of any behavioral testing. Experimenters (male and female) were the same across all experiments. The Institutional Animal Care and Use Committee approved all behavioral procedures.

Behavioral apparatus

Rats were trained and tested within 16 identical rodent observation cages (30 × 24 × 21 cm; MED Associates, Inc.) comprised of aluminum and Plexiglas. These cages are evenly distributed within two separate testing rooms in the laboratory (Room 1 and 2). The test cage floor is lined with 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (from center to center). A shock source and solid-state grid scrambler (MED Associates, Inc.) delivered footshock (unconditioned stimulus; US) to the cage floor. A speaker attached to the testing cage provided the auditory conditioned stimulus (CS). Within each observation chamber, a small fan provided background noise (~70 dB). A metal pan beneath the grid floor collected animal waste. Of note, 15 W bulbs provided lighting within each chamber as appropriate for the context (see below). Testing cages rested upon load-cell platforms, which respond to cage displacement as a result of motor activity of the subject. A load-cell amplifier sends platform activity to Threshold Activity software (MED Associates, Inc.). Load-cell activity values (−10 to +10 V) are digitized into absolute values within the Threshold Activity software; these values are multiplied by 10 to yield a range of activity of 0–100 (higher values indicate higher levels of cage displacement). Load-cell activity is digitized at 5 Hz, such that a single observation of load-cell activity is assessed every 200 msec (i.e., 300 observations per rat per minute). For all experiments, freezing behavior (i.e., immobility aside from that which is necessary for breathing) was defined as digitized load-cell values of ≤10 for two sequential seconds or longer (i.e., rats were only considered to be freezing if immobile for two or more seconds). Each load-cell is calibrated prior to the start of behavior to ensure optimal detection of motor activity and freezing behavior.

Additionally, all phases of behavioral training and testing were visually recorded from above the animals, as visible through the clear Plexiglas ceilings of the testing cages.

Contexts were made distinct by manipulating the light levels of the testing rooms, the texture of the cage floors, and the odors within the testing cages. Specifically, "Context A" consisted of ammonium hydroxide odor (50 mL of 1% ammonium hydroxide poured into the metal tray beneath the cage), testing cage lights were off, red room lights were on (white room lights were off), cage fans were off, cupboard doors of the testing chambers were closed, and subjects were shuttled in black transport boxes to and from the behavior room (Room 1). "Context B" consisted of acetic acid odor (50 mL of 1.5% acetic acid solution in the pans beneath the cage), with cage lights off, white room lights on (red room lights off), background fans on, cupboard doors open, and clear plastic cages (with sawdust bedding) for transportation of subjects. Context B utilized Room 2. "Context C" used ethanol odor (80% ethanol solution), cage lights were on, red room lights were on (white room light were off), background fans were on, solid plastic floors were placed over the grid floors of the testing cage, and solid white plastic boxes were used for transport. Context C utilized Room 2. Use of the solid plastic floors does not impair the acquisition of behavioral data. These contexts were identical across all experiments, however solid white plastic boxes were utilized to transport subjects for Context B in Experiment 1. Testing chambers were cleaned with water and wiped down with paper towels that were dipped in context odor before each behavioral squad. The steel grid floors were dried before the start of any behavior. Additionally, experimental groups were randomly assigned to a testing chamber, which was unique to each context (subjects were placed back in the same testing chamber for the same context).

Procedure

Experiment 1a/b

Rats in Experiment 1 were randomly assigned to an "exposure" group (DANGER or NEUTRAL) and an "extinction" group (EXT or NoEXT) prior to the start of behavioral training. Rats (counterbalanced by group assignments) were either tested to the CS in the ambiguous retrieval context at 30 min and 24 h (short- and long-term testing) following postextinction exposure to the dangerous or neutral context (Experiment 1a), or rats were tested at 24 h without short-term testing following the exposure phase (Experiment 1b). We performed all phases of behavioral training during the same window of time for each day. On the first day of behavioral training in Experiment 1, subjects (in squads of eight) were transported from the vivarium and placed in a distinct context for 4 min (Context A), with an unsignaled footshock (2 sec, 1 mA) delivered 3 min into the exposure (DANGER rats) or rats were merely exposed to Context A for equal duration (NEUTRAL rats). NEUTRAL rats were counterbalanced by extinction assignment and in separate squads from DANGER rats on the first day of behavioral training (data from the first day of training not shown). Twenty-four hours later, all subjects underwent auditory fear conditioning in Context B, consisting of five tone, conditioned stimulus (CS; 10 sec, 2 kHz, 80 dB tone)–footshock, unconditioned stimulus (US; 2 sec, 1 mA) pairings. The US onset occurred at the termination of the 10 sec CS. CS–US pairings were spaced along 1-min intervals, beginning 3 min after placing subjects in the chambers. Subjects remained in the test chambers for 1 min following the final CS–US pairing. All conditioning squads were counterbalanced for group assignment. Freezing (%) for conditioning was analyzed along six trials: one for baseline activity and five more trials for each minute following CS–US pairings (conditioning data not shown). Twenty-four hours after conditioning, rats underwent extinction

to the CS (EXT rats) in the conditioning context ("ambiguous") or mere exposure to conditioning context for equal duration (NoEXT rats). Specifically, following 3 min of acclimation to Context B, extinction training consisted of 2–3 d of 45 CS-only presentations (10 sec, 2 kHz, 80 dB tone), separated by 30-sec post-CS intervals. Subjects remained in Context B for 3 min following the final CS alone presentation. For NoEXT rats, subjects were exposed to Context B for an equal duration of time as for EXT rats, but without CS-only presentations. Groups were counterbalanced by exposure assignments. To efficiently represent extinction data across multiple days of training, freezing behavior was analyzed across two block trials: one block for mean freezing (%) during the first 15 post-CS intervals (or equivalent for NoEXT rats), and a second block for the final 15 post-CS intervals on the final day of extinction. Twenty-four hours after extinction training, all rats were exposed for 3 min to Context A. Rats were immediately returned to their homecages following the exposures (one trial per minute for the analyses). For rats assigned to short-term testing (Experiment 1a), subjects were brought back to the laboratory to be tested to the CS in Context B at 30 min following the exposures. Rats were given 3 min of acclimation to the testing context before the onset of five CS-only presentations, spaced by 30 sec post-CS intervals. Rats remained in the testing chamber for 3 min following the final tone presentation. In turn, each day of testing comprised of seven trials for the overall analyses: one trial for freezing at baseline, five trials for each of the 30-sec post-CS intervals, and a final trial for behavior during the remaining time in the test chamber. Twenty-four hours after short-term testing (or 24 h after the exposure phase without short-term testing; i.e., long-term-only testing [Experiment 1b]), all rats underwent testing to the CS as described for short-term testing.

Experiment 2a/b

With a novel cohort of rats, we established a dangerous or neutral context (Context A) on the first day of training for Experiment 2. DANGER rats experienced a 2 sec, 1 mA unsignaled footshock at 3 min into a 4-min exposure in Context A; NEUTRAL rats were merely acclimated to Context A for equal duration (data not shown). Twenty-four hours later, all rats were fear conditioned to an auditory CS in Context B as described for Experiment 1. Twenty-four hours after auditory fear conditioning, rats experienced either CS extinction (EXT rats) or mere exposure to Context C (NoEXT rats) over the course of 2–3 d (counterbalanced by exposure assignments). Extinction in Experiment 2 was analyzed as described in Experiment 1. Twenty-four hours after extinction training, all rats were exposed to Context A for 3 min then returned to their homecages for either 30 min (short-term testing; Experiment 2a) or 24 h (long-term-only testing; Experiment 2b). Testing in Experiment 2 (both short- and long-term) followed the same procedures for behavior (and analyses, where appropriate) as described for Experiment 1, except rats were tested in Context C, which served as the "safe" context by our terms.

Experiment 3

Untrained subjects in Experiment 3 underwent fear conditioning in Context B on the first day of behavioral training, without the prior establishment of a separate dangerous or neutral context. Fear conditioning in Experiment 3 was procedurally identical to Experiments 1 and 2 (data not shown). Extinction to the CS (EXT rats) occurred over 2 d in Context C (analyses were identical to Experiments 1 and 2). Mere exposure for NoEXT rats occurred in Context C for equal duration. Twenty-four hours after the extinction phase, rats were exposed to either the

conditioning context (Context B) for 3 min (DANGER rats), or the extinction context (Context C) for 3 min (SAFE rats). Rats were returned to the vivarium immediately after the exposure phase. Thirty minutes later, all rats were tested to the CS in Context C. An identical test occurred 24 h later.

Data analysis

Freezing behavior served as the index of fear throughout all phases of the study. Freezing behavior was defined as the percentage of total time spent immobile during each trial or block of trials as indicated above. All data were analyzed with ANOVAs followed by post hoc comparisons (Fisher's Protected Least Significant Difference test) after a significant omnibus *F*-ratio. No rats were excluded from the analyses.

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CHAPTER III

BNST MEDIATES REINSTATEMENT BUT NOT RENEWAL OF FEAR***

Introduction

Fear relapse plagues clinical interventions for fear-related anxiety disorders (Hooley, 2007; Boschen et al., 2009; Vervliet et al., 2013b). Various factors—such as the nature of the therapeutic intervention, duration of time since treatment, and intervening stress—have been shown to be important in determining the degree of retention of extinguished fear in humans and other animals (Kehoe and Macrae, 1997; Bouton, 2002, 2014; Myers and Davis, 2002; Bouton et al., 2006; Hermans et al., 2006; Fitzgerald et al., 2014; Goode and Maren, 2014; Luck and Lipp, 2015). Pavlovian fear conditioning and extinction in rodents provides a clinically relevant model to explore the behavioral and brain mechanisms of relapse. Specifically, fear conditioning in rats is a behavioral procedure through which subjects experience concomitant pairings of a neutral conditioned stimulus (CS), such as a tone, with an aversive unconditioned stimulus (US), such as a footshock (Rescorla, 1988a,b). After fear conditioning, presentation of the CS alone comes to elicit conditioned fear responses (CRs), including freezing behavior (Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001). Fear CRs also occur in the place or "context" in which fear conditioning was experienced (Bouton and King, 1983; Maren et al., 2013).

***Reprinted with permission from "Reversible inactivation of the bed nucleus of the stria terminalis prevents reinstatement but not renewal of extinguished fear" by Goode T.D., Kim J.J., & Maren, S., 2015. *eNeuro*, 2, ENEURO.0037-15.2015.

After conditioning, repeated presentations of the CS in the absence of footshock lead to the extinction of fear (Pavlov, 1927; Chang et al., 2009). It is widely believed that research on fear extinction in rodents can enhance our understanding of exposure therapy in humans (Barad, 2005; Milad et al., 2006, 2014; Morrison and Ressler, 2014). Extensive research indicates that extinction training does not necessarily erase fear memory; rather, it results in a new "inhibitory" memory that limits the expression of the fear (Konorski, 1967; Bouton, 2004; Maren, 2011). Consequently, extinction memories are susceptible to relapse. Two forms of fear relapse have received considerable attention over the years: "reinstatement" and "renewal." Reinstatement of fear occurs when an aversive, unsignaled US is experienced prior to presentation of the extinguished CS (Rescorla and Heth, 1975; Bouton and Bolles, 1979). Reinstatement is most robust in contexts in which reinstating shocks are delivered, although it can also occur in contexts never paired with shock (Westbrook et al., 2002; see also Morris et al., 2005a; Halladay et al., 2012; Goode et al., 2015). This suggests that reinstatement can be mediated by either direct context–US associations (Bouton and King, 1983; Bouton et al., 2006) or though stress states that generalize across contexts (Haroutunian and Riccio, 1977; Morris et al., 2005b; Deschaux et al., 2013). Fear renewal, on the other hand, occurs when a CS is presented outside of its extinction context (Bouton and Bolles, 1979; Polack et al., 2013; Vervliet et al., 2013a). Importantly, renewal does not require that the animal experience the US after extinction. Indeed, direct context–US associations do not mediate renewal (Bouton and Ricker, 1994; Harris et al., 2000; Corcoran and Maren, 2004).

The different roles that context plays in reinstatement and renewal suggest that distinct neural circuits mediate them. One brain area that has been implicated in reinstatement is the bed nucleus of the stria terminalis (BNST). In particular, BNST lesions impair the shock-induced

reinstatement of fear (Waddell et al., 2006; see also Waddell et al., 2008). Sustained fear responses to conditioned contexts (Sullivan et al., 2004) and long-duration CSs (Waddell et al., 2006) also appear to rely on the BNST (also, see Walker and Davis, 1997). Conversely, BNST manipulations do not affect fear to short-duration CSs paired with shock (LeDoux et al., 1988; Sullivan et al., 2004; Waddell et al., 2006; Zimmerman and Maren, 2011). Coinciding with this evidence, BNST circuitry is also involved in the stress-induced reinstatement of drug seeking. For example, antagonism of corticotropin-releasing factor receptors within the BNST blocks the reinstatement of cocaine seeking after footshock exposure (Erb and Stewart, 1999). Similarly, pharmacological inactivation of the BNST prevents the stress-induced reinstatement of cocaine seeking following systemic administration of the anxiogenic drug yohimbine (Buffalari and See, 2011). Collectively, these data suggest that the BNST may have a selective role in forms of relapse that depend on stress and/or contextual fear, such as in reinstatement. To explore this question, we examined the consequences of reversibly inactivating the BNST in the expression of both the reinstatement and renewal of fear after extinction in rats. We hypothesized that BNST inactivation would attenuate the reinstatement, but not the renewal, of extinguished fear.

Results

Histology

Injection sites within the BNST are illustrated in Figure 5. For Experiment 1, 16 injectors terminated within the anterior lateral division of the BNST (which includes the anterolateral area, juxtacapsular nucleus, oval nucleus, and rhomboid nucleus), 4 were localized to the anterior medial BNST (which includes the anterodorsal area and central core of the anterodorsal area), 5

were localized to the anterior ventral BNST (which includes the anteroventral area, dorsolateral nucleus, dorsomedial nucleus, fusiform nucleus, magnocellular nucleus, subcommissural zone, and ventral nucleus), and 9 were located in the anterior commissure within the anterior BNST (Swanson, 1998). This yielded the following groups for the final analyses: MUS, $n = 7$; VEH, $n = 10$. Cannulae missed their targets in 15 animals; these animals (MUS, $n = 9$; VEH, $n = 6$) were analyzed separately to determine whether off-target drug infusions affected reinstatement.

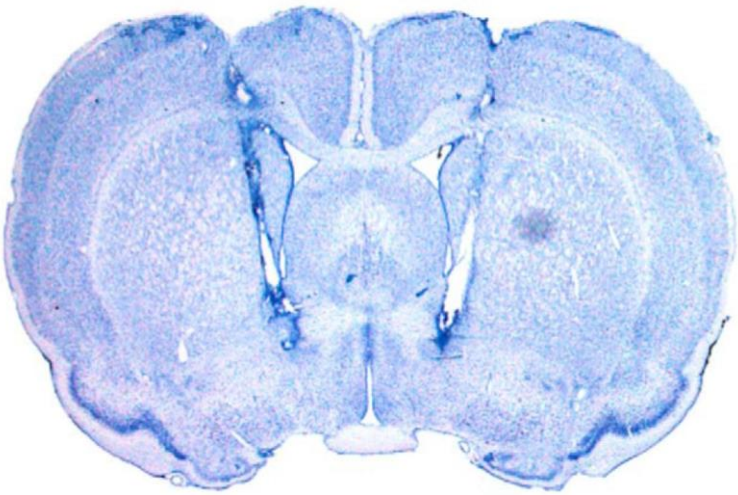

Figure 4. Representative photomicrograph of a thionin-stained coronal section from the brain of a rat with injector tips terminating within the bed nucleus of the stria terminalis.

For Experiment 2, 45 animals received bilateral injectors within the BNST. Of these animals, 10 subjects were excluded based on the behavioral criteria described above, yielding the following groups: DRUG/DIFF, $n = 11$; DRUG/SAME, $n = 6$; VEH/DIFF, $n = 8$; and VEH/SAME, $n = 10$. Accordingly, 70 injection sites from 35 animals are illustrated in Figure 5. Thirteen injectors were localized to the anterior lateral division of the BNST, 4 terminated within the anterior medial division, 22 terminated within the anterior ventral division, 5 terminated in

the anterior commissure within the anterior BNST, and 26 terminated within the posterior division of the BNST (which includes the interfascicular nucleus, principal nucleus, and transverse nucleus).

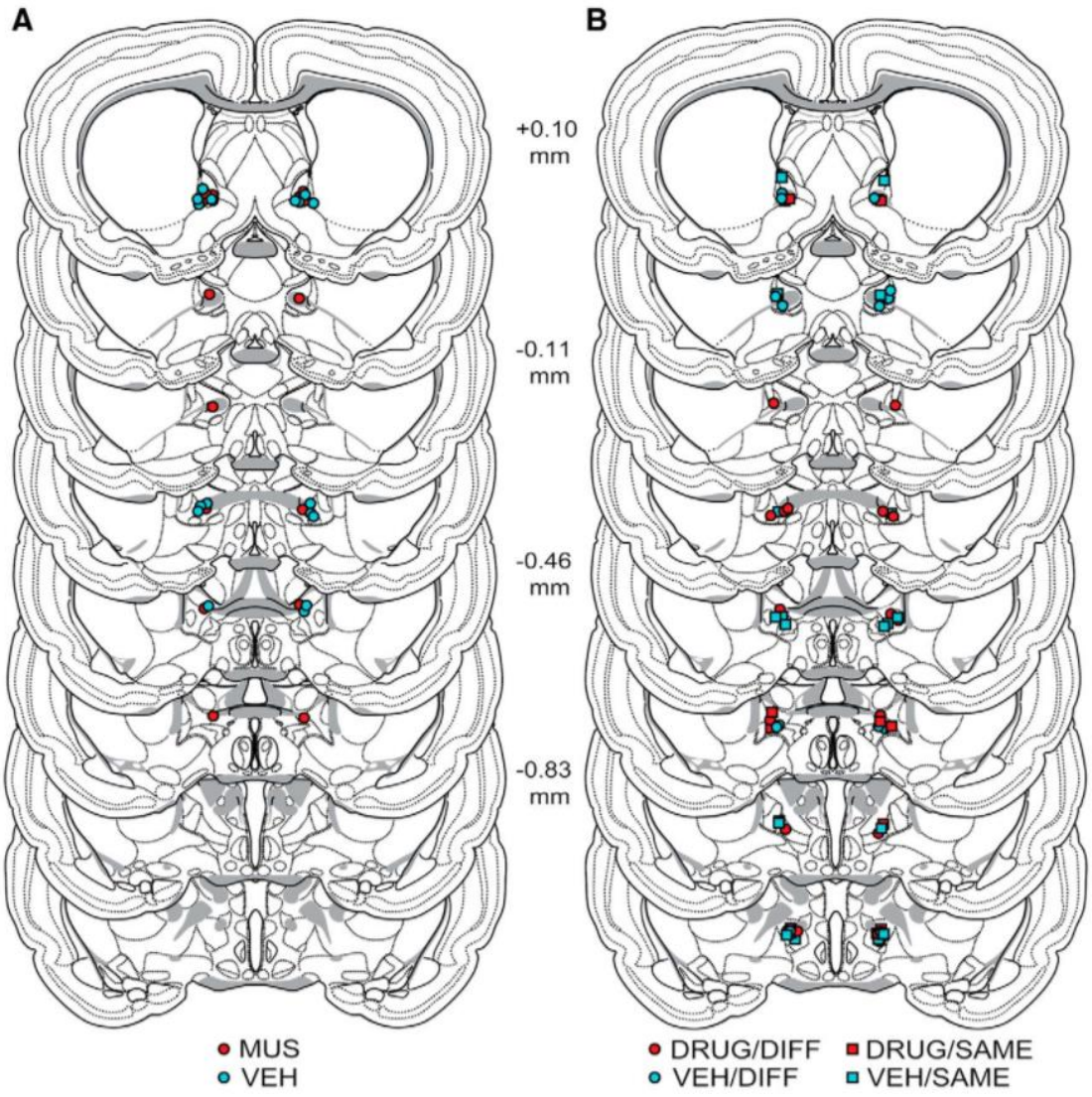

Figure 5. Illustration of cannula placement sites in the bed nucleus of the stria terminalis. Placements are shown for all rats included in the final analyses for Experiment 1 **(A)** and Experiment 2 **(B)**. Adapted from Swanson (1998). Distances shown are relative to bregma.

Experiment 1: BNST inactivation prevents reinstatement of fear to an extinguished CS

Rats exhibited reliable fear conditioning to the auditory CS (Fig. 6). This impression was confirmed in an ANOVA by a significant main effect of trial ($F_{(5,75)} = 9.1$; $p < 0.0001$[a]); freezing behavior increased across the conditioning session, and there were no group differences on this measure (*F*values < 1). Over the next 2 d, all rats were extinguished to the CS in Context A. During the first extinction session (Fig. 6), there was a significant main effect of trial ($F_{(10,150)} = 10.6$; $p < 0.0001$[b]) as freezing behavior decreased over the course of the extinction session; there were no group differences in extinction rate or magnitude (*F* values < 1). During the second extinction session (Fig. 6), there again was a significant main effect of trial ($F_{(10,150)} = 7.9$; $p < 0.0001$[c]), reflecting decreases in freezing behavior over the course of the session; again, there were no group differences in extinction rate or magnitude (*F* values < 1). On Day 4, all rats received an unsignaled footshock to reinstate fear to the extinguished CS (Fig. 6). Freezing behavior reliably increased after footshock. This impression was confirmed in an ANOVA that revealed a significant main effect of trial for freezing across the preshock and postshock periods ($F_{(1,15)} = 49.5$; $p < 0.0001$[d]). Shock-induced increases in fear on Day 4 were similar across drug and context conditions (*F* values < 2).

Twenty-four hours after the reinstatement shock, rats were infused with muscimol or vehicle into the BNST and immediately placed in Context B for a CS retrieval test. During the 10 min baseline prior to the first CS presentation, vehicle-treated rats exhibited significantly greater levels of freezing than muscimol-treated animals (VEH, 31.108 ± 5.644%; MUS, 3.968 ± 1.409%). This was confirmed in the ANOVA by a significant main effect of drug across baseline freezing ($F_{(1,50)} = 15.423$; $p = 0.0013$[e]). In addition, and as shown in Figure 4, VEH-treated rats

exhibited significantly greater levels of fear to the extinguished CS than MUS-treated animals. This impression was confirmed in the ANOVA by a main effect of drug across all testing trials ($F_{(1,90)} = 14.446$; $p = 0.0017$[f]). There was a significant main effect of trial ($F_{(6,90)} = 5.737$; $p < 0.0001$[g]) insofar as freezing behavior increased on average after presentation of the CS. Freezing to the CS during the retrieval test in vehicle-treated rats was significantly greater than during the final block of extinction, indicating successful reinstatement of extinguished fear ($F_{(1,9)} = 14.607$; $p = 0.0041$[h]). Importantly, reinstatement impairments were obtained only in rats with cannula placements in the BNST. Muscimol infusion in rats with off-target placements that missed the BNST exhibited normal reinstatement and did not differ from controls during either the baseline or CS periods (F values < 0.5). Overall, these data reveal that BNST inactivation reduced both contextual freezing and the reinstatement of fear to an extinguished CS.

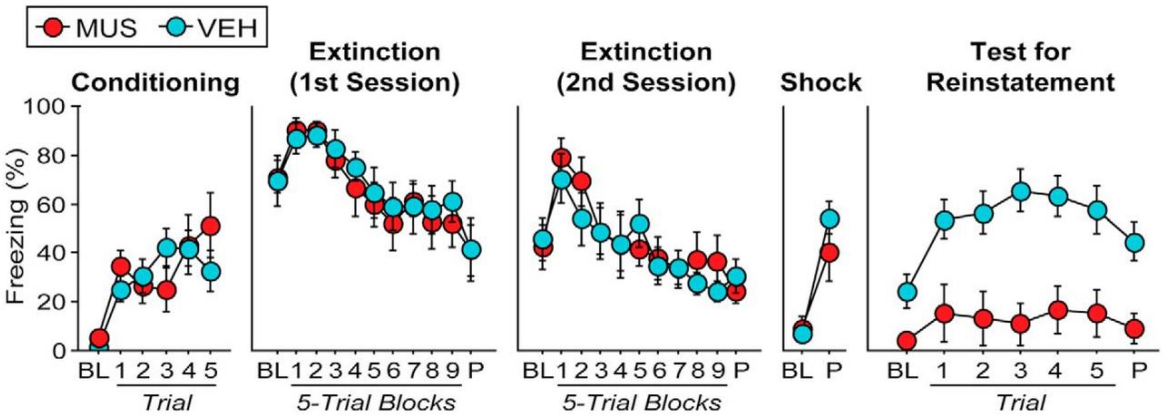

Figure 6. Pharmacological inactivation of the BNST prevents reinstatement. Conditioning, Mean (±SEM) percentage of freezing during the 3 min baseline (BL) and in the 60 s interstimulus interval following each CS–US pairing. Extinction (First Session), Mean (±SEM) percentage freezing during a 3 min BL and across nine extinction blocks (each block represents average responding during the 30 s post-CS intervals after five extinction trials). The rats remained in the chambers for 150 s after the final CS presentation (P). Extinction (Second Session), Mean (±SEM) percentage freezing for the second day of extinction training (trials are equivalent to the first extinction day). Shock, Mean (±SEM) percentage freezing during the 3 min BL period before unsignaled footshock and during the 1 min postshock period. Test for Reinstatement, Mean (±SEM) percentage freezing during the final 3 min of the BL period immediately prior to CS onset and during five 30 s interstimulus intervals after each test trial; the rats remained in the chambers for 150 s after the final CS.

Experiment 2: BNST inactivation does not alter the expression of fear renewal

As shown in Figure 7, rats exhibited reliable fear conditioning. This impression was confirmed in the ANOVA by a significant main effect of trial ($F_{(5,155)}$ = 31. 2; $p < 0.0001$[i]); freezing behavior increased over the course of conditioning, and the groups did not differ from one another (*F* values < 1). Twenty-four hours later, rats significantly reduced their fear across extinction trials (Fig. 7; main effect of trial, $F_{(10,310)} = 50.6$; $p < 0.0001$[j]). Extinction of fear on Day 2 was similar across group assignments (*F* values < 1).

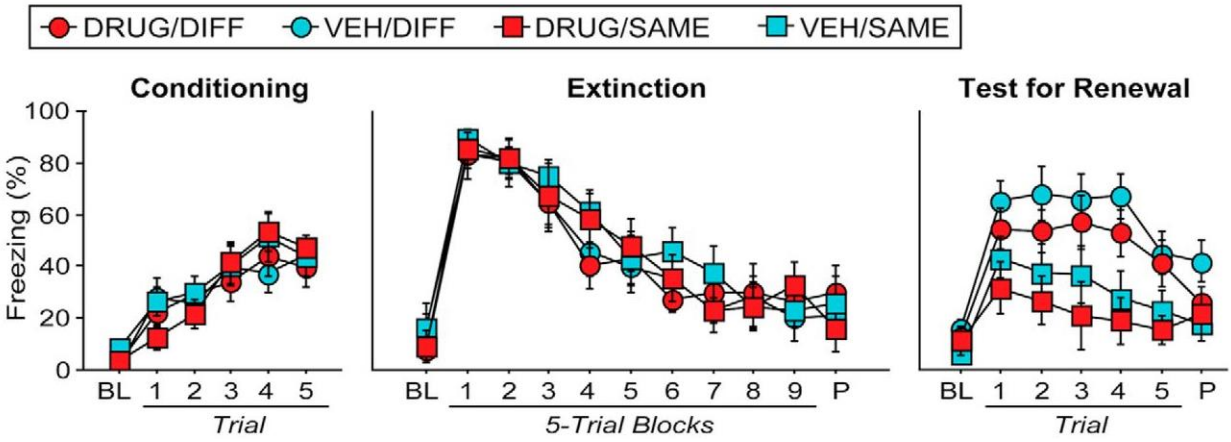

Figure 7. Pharmacological inactivation of the BNST does not prevent renewal. Conditioning, Mean percentage of freezing during the 3 min baseline (BL) and in the 60 s interstimulus interval following each CS–US pairing. Extinction, Mean (±SEM) percentage freezing during a 3 min BL and across nine extinction blocks. Each block represents the average responding during the 30 s post-CS intervals after five extinction trials. The rats remained in the chambers for 150 s after the final CS presentation (P). Test for Renewal, Mean (±SEM) percentage freezing during the 3 min baseline period immediately prior to CS onset and during five 30 s interstimulus intervals after each test trial; the rats remained in the chambers for 150 s after the final CS.

On Day 3, rats were infused with either drug or vehicle immediately before receiving a retrieval test in the extinction context (SAME) or in the context in which extinction did not occur (DIFF). As indicated in Figure 7, both VEH- and DRUG-treated rats exhibited robust fear renewal in the DIFF context relative to the low levels of freezing expressed by rats in the SAME

context. BNST inactivation did not attenuate the renewal of fear to the extinguished CS. This impression was confirmed in the ANOVA by a main effect of context ($F_{(1,186)}$ = 12.843; p = 0.0011[k]) such that rats in the DIFF condition exhibited significantly more freezing across test trials than those in the SAME condition (regardless of drug condition). A significant trial × context interaction ($F_{(6,186)}$= 2.647; p = 0.0173[l]) indicated that DIFF rats exhibited greater freezing after CS onset compared to SAME rats. Moreover, DIFF rats (but not SAME rats; F values < 0.5) exhibited significantly more freezing across test trials relative to the final block of extinction ($F_{(1,18)}$ = 16.005; p = 0.0008[m]), indicating robust renewal of fear. Hence, BNST inactivation did not impair either the renewal or expression of fear to an extinguished auditory CS.

In-text letter	Data structure	Type of test	Power
a	Normal distribution	One-way repeated-measures ANOVA (conditioning trials)	1.000
b	Normal distribution	One-way repeated-measures ANOVA [extinction trials (Day 2)]	1.000
c	Normal distribution	One-way repeated-measures ANOVA [extinction trials (Day 3)]	1.000
d	Normal distribution	One-way repeated-measures ANOVA (reinstating shock trials)	1.000
e	Normal distribution	One-way repeated-measures ANOVA (infusion group across baseline at test)	0.968
f	Normal distribution	One-way repeated-measures ANOVA (infusion group across testing)	0.957
g	Normal distribution	One-way repeated-measures ANOVA (test trials)	0.993
h	Normal distribution	One-way repeated-measures ANOVA (final extinction block vs mean responding at test for VEH animals)	0.935
i	Normal distribution	One-way repeated-measures ANOVA (conditioning trials)	1.000
j	Normal distribution	One-way repeated-measures ANOVA (extinction trials)	1.000
k	Normal distribution	One-way repeated-measures ANOVA (renewal group across testing)	0.951
l	Normal distribution	Two-way repeated-measures ANOVA (testing trials × renewal group)	0.858
m	Normal distribution	One-way repeated-measures ANOVA (final extinction block vs mean responding at test for DIFF animals)	0.977

Table 2. Statistical table.

Discussion

The current study reveals the novel finding that the BNST plays a specific role in the shock-induced reinstatement of extinguished fear; BNST inactivation did not affect the renewal of extinguished fear that accompanies a change in context. Deficits in the reinstatement of

extinguished fear were paralleled by reductions in the expression of contextual freezing after BNST inactivation. These results are consistent with an earlier report (Waddell et al., 2006) revealing that neurotoxic lesions of the BNST impair the shock-induced reinstatement of extinguished fear. Additionally, our work parallels findings revealing that the BNST is necessary for shock-induced reinstatement of extinguished drug-seeking behavior (Erb and Stewart, 1999; Erb et al., 2001; see also Leri et al., 2002; Buffalari and See, 2011). Collectively, these data suggest that the BNST has a critical role in the relapse of extinguished behaviors caused by the experience of aversive stimuli (for review, see Smith and Aston-Jones, 2008; Silberman and Winder, 2013; Stamatakis et al., 2014).

Although BNST inactivation impaired fear reinstatement, it did not affect fear renewal despite the fact that the extent of relapse was similar between experiments. This reveals that deficits in reinstatement are not due to impairments in the expression of freezing per se. Indeed, this pattern of results is consistent with other reports indicating that the BNST has a selective role in the expression of fear to contextual compared to discrete CSs (LeDoux et al., 1988; Walker and Davis, 1997; Sullivan et al., 2004; Waddell et al., 2006, 2008; Zimmerman and Maren, 2011; Sink et al., 2013; see also Duvarci et al., 2009; Haufler et al., 2013). Importantly, pretraining lesions of the BNST do not disrupt the acquisition of conditioned fear to discrete CSs (LeDoux et al., 1988; Waddell et al., 2006), nor do post-training lesions of the BNST affect the expression of conditioned fear to discrete CSs (Sullivan et al., 2004). However, BNST lesions attenuate the expression of fear to shock-associated contexts (Sullivan et al., 2004) and attenuate the expression of fear responses to long-duration CSs (i.e., 10 min tones paired with shock; Waddell et al., 2006). Additionally, Sullivan et al. (2004) reported that rats with BNST lesions exhibited blunted corticosterone responding during exposure to a conditioned

context (see also Resstel et al., 2008). Hence, it is believed that BNST inactivation prevents reinstatement by reducing the expression of contextual fear, which is thought to be essential for the reinstatement effect (Bouton and Bolles, 1979; Westbrook et al., 2002; Bouton et al., 2006; Waddell et al., 2006).

A key finding in the present study is that BNST inactivation did not affect fear renewal. Unlike reinstatement, however, renewal does not require contextual fear. Indeed, fear renewal can be obtained in contexts that have never hosted shock (e.g., "ABC" or "AAB" renewal; Bouton and Bolles, 1979; Bouton and Ricker, 1994; Harris et al., 2000; Westbrook et al., 2002; Corcoran and Maren, 2004; Holmes and Westbrook, 2013; Jin and Maren, 2015). Renewal also occurs in shock-associated contexts that have themselves undergone extinction and no longer support contextual fear (e.g., "ABA" renewal; Bouton and King, 1983; Vansteenwegen et al., 2005; Effting and Kindt, 2007; Knox et al., 2012; Polack et al., 2013; Holmes and Westbrook, 2014). These findings support the idea that renewal depends not on direct context–US associations, but on a contextual retrieval process that informs the animal of what a CS means in a particular context (Bouton and Bolles, 1979; Bouton and King, 1983; Bouton and Peck, 1989; Holland, 1992; Bouton, 1993; Bouton and Ricker, 1994; Harris et al., 2000; Bouton et al., 2006; Ji and Maren, 2007; Maren et al., 2013; Vervliet et al., 2013b; Delamater and Westbrook, 2014). Importantly, the present results strengthen this view insofar as renewal of fear was immune to BNST inactivation (a manipulation that impairs contextual fear). Considerable work now reveals that this contextual retrieval process depends on a circuit involving the amygdala, hippocampus, and prefrontal cortex (Corcoran and Maren, 2004; Ji and Maren, 2005, 2007; Herry et al., 2008; Knapska and Maren, 2009; Orsini et al., 2011, 2013; Zelikowsky et al., 2012; Maren, 2014; Jin and Maren, 2015).

An important issue that has yet to be resolved is which subregions of the BNST contribute to the effects we have observed in the present study. Indeed, the BNST is heterogeneous in structure, and different subregions within the BNST appear to make unique contributions to fear and anxiety (Walter et al., 1991; Dong et al., 2001a; Dong and Swanson, 2003, 2004a,b; Choi et al., 2007; Jennings et al., 2013; Kim et al., 2013). For example, Kim et al. (2013) showed that photostimulation of the oval nucleus of the BNST resulted in anxiety-related behaviors, while photostimulation of the anterodorsal region of the BNST resulted in anxiolytic behaviors. Jennings et al. (2013) demonstrated that photostimulation of glutamatergic projections of the ventral BNST to the ventral tegmental area (VTA) produced increases in anxiety, whereas photostimulation of GABAergic BNST projections to the VTA was anxiolytic. In the present study, cannula placements terminated primarily within the anterior portion of the BNST (particularly the anterior lateral and anterior ventral divisions of the BNST), though several rats received infusions within the posterior division of the BNST in Experiment 2. The spread of drug likely affected multiple BNST nuclei in these areas. Circuit-selective chemogenetic or optogenetic techniques would help to clarify the specific BNST subregions contributing to fear reinstatement (Sparta et al., 2013).

In humans and other animals, the BNST shares connections with several important emotion-regulating regions in the brain, including the amygdala, dorsal raphe nucleus, hippocampus, hypothalamus, nucleus accumbens, prefrontal cortex, and ventral tegmental area (Swanson and Cowan, 1977; Weller and Smith, 1982; Phelix et al., 1992; Sun and Cassell, 1993; Dong et al., 2001a,b; Dong and Swanson, 2003, 2004a,b; Jalabert et al., 2009; Crestani et al., 2013; Avery et al., 2014; Roman et al., 2014; Krüger et al., 2015). Not surprisingly, the BNST has been implicated in various depression- and anxiety-related behaviors (for review,

see Walker and Davis, 2008; Hammack et al., 2009, 2010,2012; Walker et al., 2009; Davis et al., 2010; McElligott et al., 2013; Adhikari, 2014; Kash et al., 2015). Importantly, the BNST modulates hypothalamic–pituitary–adrenal axis activity, including corticosterone release, via its connections with the hypothalamic paraventricular nucleus (Cullinan et al., 1993; Herman et al., 1994; Sullivan et al., 2004; Choi et al., 2007; Crestani et al., 2013). Corticosterone release is correlated with both the acquisition and expression of conditioned fear (Campeau et al., 1997; Pugh et al., 1997; Cordero et al., 1998; Roozendaal et al., 2006; Marchand et al., 2007). Hence, BNST lesions might influence reinstatement by limiting the modulatory effects of corticosterone on fear expression to an extinguished CS. Alternatively, the BNST is positioned to directly influence freezing behavior via its projections to the amygdala and periaqueductal gray (Dong et al., 2001a; Dong and Swanson, 2003, 2004a,b; Fendt et al., 2003; Asok et al., 2013). In this way, the BNST might directly drive reinstatement of fear to an extinguished CS by driving amygdaloid and periaqueductal gray circuits involved in fear expression. In either case, BNST-mediated modulation of contextual fear might summate with fear to the extinguished CS to yield reinstatement.

In conclusion, the present results reveal that distinct neural circuits mediate different forms of fear relapse. Here we show that the BNST is especially important for reinstatement, a form of relapse produced by the exposure of animals to aversive stimuli. Hence, selective manipulations of the BNST may be particularly effective in preventing fear relapse in aversive contexts. Ultimately, appreciating the circumstances that give rise to the return of fear will help in isolating circuit-specific therapies for combating fear relapse.

Materials and methods

Subjects

All subjects were adult (200-250 g) male Long−Evans (Blue Spruce) rats from Harlan Laboratories. Upon arrival, rats were individually housed in clear plastic cages on a rotating cage rack (Animal Care Systems). Group assignments for behavioral training were randomized for cage position on the racks. Rats were given free access to standard rodent chow and water. Sawdust served as bedding for the rats (bedding was changed once a week). Behavioral experiments took place on different days from the days that cages were changed. Rats were kept on a fixed light/dark cycle, with rats experiencing 14 h of light (starting at 7:00 A.M.) followed by 10 h of darkness each day. All handling, surgeries, and behavioral testing occurred during the light hours of the light/dark cycle. The experimenters handled each rat for 1 min/d for 5 d prior to the start of surgeries. Additionally, rats were habituated to the infusion procedures and to the infusion room prior to behavioral training. The Texas A&M University Institutional Animal Care and Use Committee approved all experimental procedures.

Surgery

Rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and were treated with atropine methyl nitrate (0.4 mg/kg, i.p.). After the induction of anesthesia, the head of each rat was shaved, and the rats were placed in a stereotaxic frame (David Kopf Instruments). The scalp was incised, and the skull was leveled, with bregma and lambda in the same horizontal plane. Small holes were drilled in the skull and steel guide cannulae (26 gauge, 8 mm; Small Parts) were lowered into the BNST (0 mm anteroposterior to bregma, ±2.7 mm mediolateral, and −5.9 mm ventral to dura). Guide cannulae were angled at 10° to limit penetration of the lateral ventricles. Three stainless steel screws were affixed to the

skull, and the entire skull surface was covered with dental cement to secure the cannulae to the skull. Stainless steel obturators (30 gauge, 9 mm; Small Parts) were placed inside each cannula and changed every 2 d prior to behavioral testing. Rats were given a single bacon-flavored Rimadyl tablet (2 mg/tablet; Bio-Serv) following surgery. The rats were allowed at least 1 week to recover from surgery before behavioral testing.

Behavioral apparatus

Behavioral testing was conducted in two distinct rooms within the laboratory ("Wellborn" and "University"). Each testing room contained eight identical conditioning chambers (Med Associates) fabricated with aluminum (sidewalls) and Plexiglas (rear wall, ceiling, and front cage door) walls (30 × 24 × 21 cm). The conditioning chambers were housed in external sound-attenuating cabinets. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter); each rod was spaced 1.5 cm apart (center to center). Each chamber was equipped with a speaker to provide the CS. As described for each context (see below), a 15 W house light provided ambient lighting and a cabinet fan provided background noise for each chamber (~70 dB). The grid floors of each chamber were connected to a shock source and a solid-state grid scrambler to deliver the footshock US (Med Associates). Each chamber rested on a load-cell platform that detected chamber displacement in response to the movement of each animal. Load-cell activity values (range, −10 to +10 V) were acquired during all behavioral phases and digitized at 5 Hz with Threshold Activity Software (Med Associates). Load-cell output was transformed off-line to values ranging from 0 to 100 (higher values indicate more displacement of the cage). A bout of freezing was scored if the absolute values of load-cell activity were ≤10 for ≥1 s (Maren, 1998). The number of 1 s bins of freezing was divided by the

total number of bins in each observation period (typically, a 30 s or 1 min period after each trial) to yield the percentage of time each animal was freezing.

Distinct contexts were created through the use of different odors and visual cues. For Experiment 1, conditioning and extinction occurred in Context A; these chambers were located in the Wellborn test room in the laboratory. For Context A, the cage walls were wiped with acetic acid (1.5%) and a small volume was placed in the trays underneath the grid floor. The houselights were extinguished, but the overhead fluorescent room lights were illuminated. Rats were transported to Context A in white containers, and the cabinet doors enclosing the conditioning chambers were open during testing. The reinstatement sessions occurred in Context B; these chambers were located in the University test room in the laboratory. For Context B, ammonium hydroxide (1%) was used to wipe the cage walls and a small volume was placed in the trays underneath the grid floors. The overhead fluorescent room lights remained off (red room lights provided overhead illumination); the houselights within each testing chamber were illuminated. Rats were transported to Context B in black containers, and the cabinet doors enclosing the conditioning chambers were closed during testing. Cabinet fans were turned on for both Context A and B in Experiment 1. For Experiment 2, conditioning was conducted in Context A as described above, whereas extinction and renewal testing used Contexts B and C (per group assignments; cabinet fans were turned off for Contexts B and C in Experiment 2). For Context C (Wellborn room), ethanol (70%) was used to wipe the cage walls and a small volume was placed in the trays beneath the grid floor. The houselights were illuminated, and the overhead lights were extinguished; a thin black Plexiglas sheet covered the grid floor for Context C. Cabinet doors enclosing the chambers were open during testing in Context C. Rats were

transported to Context C in white 5 gallon buckets; a layer of sawdust was placed in each bucket and changed out for each squad of animals.

Behavioral procedures

Experiment 1: effects of BNST inactivation on the expression of reinstatement

An illustration of the behavioral paradigm for Experiment 1 is shown in Figure 8. Prior to behavioral testing, 32 rats were randomly assigned to groups that would receive intracranial infusions of either muscimol (MUS; a selective $GABA_A$ receptor agonist; $n = 16$) or vehicle (VEH; physiological saline; $n = 16$) prior to retrieval testing. MUS rats received a total of 0.3 μg of muscimol (1.0 μg/μl in 0.3 μl) per hemisphere. VEH rats were infused with 0.3 μl of physiological saline per hemisphere. All infusions occurred over 1 min at a rate of 0.3 μl/min. Within each drug condition, rats were also randomly assigned to receive reinstatement shock in either the test context (Context B) or the extinction context (Context A). Rats did not differ at test based on the context in which the reinstatement shock was delivered (F values < 1); therefore, we collapsed rats across this condition.

On Day 1, rats were transported to Context A for fear conditioning. Three minutes after placement in the chambers, rats received five auditory CSs (10 s, 2 kHz, 80 dB)–footshock US (2 s, 1 mA) pairings [US onset occurred upon CS offset; 70 s intertrial intervals (ITIs)]. After the final conditioning trial, rats remained in the chambers for 1 min before being returned to their home cages. Twenty-four hours later, rats underwent the first of two extinction sessions. During these sessions, they were returned to the conditioning context (Context A), and, after 3 min, rats were presented with 45 CS-alone trials (40 s ITIs). After the final CS presentation, the rats remained in the chambers for 3 min before being returned to their home cages. The second

extinction session was identical to the first and occurred on the following day. Twenty-four hours after the final extinction session, rats underwent a reinstating shock session. First, rats were exposed to either Context A or B for 4 min in the absence of the CS or US; rats were returned to their home cages after this experience. Two hours later, rats were brought back to the laboratory and were placed in the other context (A or B; per group assignments) for a reinstating shock in that context. For the reinstating shock session, rats received a single, unsignaled footshock (1 s, 0.4 mA) after 3 min in chambers. Rats remained in the chambers for 1 min after shock offset. Last, on Day 5, and immediately prior to retrieval testing, the rats were infused with muscimol or vehicle and transported to Context B to assess fear to the extinguished CS. Ten minutes after placement in Context B, the rats received five CS-only presentations (40 s ITIs). Rats remained in the testing chambers for 3 min following the final CS presentation.

Experiment 2: effects of BNST inactivation on the expression of renewal

Refer to Figure 8 for an illustration of the behavioral paradigm used for Experiment 2. Seventy-six rats were randomly assigned to drug (DRUG or VEH) and testing [different (DIFF) or SAME] conditions. Immediately prior to renewal testing, DRUG rats were infused with either 0.3 μg of muscimol (1.0 μg/μl in 0.3 μl) per hemisphere (identical to Experiment 1) or 3.0 μg of 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX; 10.0 μg/μl in 0.3 μl) per hemisphere. NBQX is a potent AMPA receptor and kainate receptor antagonist. We found no difference in the effects of NBQX or muscimol on conditional freezing at test (*F* values < 0.1); therefore, we collapsed DRUG rats across this condition. Rats assigned to receive VEH were infused with 0.3 μl of physiological saline. As in Experiment 1, all infusions were delivered at 0.3 μl/min for 1 min. Rats assigned to the SAME condition were tested to the extinguished CS in

the extinction context, whereas rats assigned to the DIFF condition experienced the extinguished CS outside of the extinction context (but in a familiar context).

On Day 1, all rats were conditioned with five CS–US pairings (CS: 10 s, 2 kHz, 80 dB auditory tone; US: 2 s, 1 mA footshock) in Context A (the procedure was identical to Experiment 1). Twenty-four hours later (Day 2), rats were first exposed for 35 min to the context (either Context B or Context C) that was not hosting extinction; this ensured that exposure to all contexts was counterbalanced. Three hours later, the rats were extinguished in the alternate context (either Context B or Context C; counterbalanced by group). Three minutes after placement in the extinction context, the rats received 45 CS-only presentations (40 s ITIs); the rats remained in the chambers for 3 min after the final CS presentation. Twenty-four hours later, and immediately prior to renewal testing, rats were infused with either drug (muscimol or NBQX) or VEH and transported to the appropriate test context (which was either the same as or different from the extinction context). Responding at the test was not affected by whether the renewal context was B or C (F values < 0.1); the data are collapsed across this condition. Three minutes after placement in the test context, rats received five CS-only presentations (40 s ITIs). Rats remained in the testing chamber for 3 min after the final CS-only presentation.

Intracranial infusions

Rats were transported in 5-gallon buckets to a procedure room within the colony for drug infusions. The obturators were removed from the guide cannulae and stainless steel injection needles (33 gauge, 9 mm; extending 1 mm beyond the end of the guide) were inserted. Each injector (Small Parts) was attached to polyethylene tubing (PE-20; Braintree Scientific), which in turn was connected to a gastight 10 µl syringe (Hamilton, Co.). Syringes were mounted in an infusion pump (KD Scientific). After insertion of the injectors, the rats were returned to the

buckets where they remained unrestrained during the infusion procedure. After the infusion, the injection needles remained in the guide cannulae for 1 min before being removed; clean obturators were inserted into the guides, and the rats were transported to the conditioning chambers.

Histological procedures

Within 1 week after the final retention test, the rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml, i.p.) and perfused. Transcardial perfusions were performed with physiological saline followed by 10% formalin solution. Brains were removed from the skull and stored in 10% formalin for 24 h at 4° C followed by 30% sucrose-formalin for at least 3 d before sectioning. Brain tissue was flash frozen with dry ice and sectioned at 40 μm on a cryostat (Leica Microsystems) at −20° C. Sections were wet mounted to microscope slides and stained with 0.25% thionin to identify cannula tracts and to localize injection sites in the tissue. Photomicrographs of the sections (10× magnification) were captured and digitized using a Leica MZFLIII microscope. Figure 2 shows a representative coronal section from a rat with injector tips localized to the BNST.

Data analyses

Freezing served as the index of fear for all behavioral analyses. All data were submitted to ANOVA (in-text lowercase superscripts correspond to the analyses in Table 2). *Post hoc* comparisons (Fisher's protected least significant difference test) on individual group means were calculated after a significant omnibus *F* ratio in the ANOVA; α was set at 0.05. Rats were excluded from the analyses if they failed to extinguish by the final extinction session (mean

freezing, >50%) or if mean pre-CS freezing during the retrieval test was >50%. Based on these criteria, 10 rats were excluded from Experiment 2. Unless noted otherwise, freezing data (as a percentage of the total time spent immobile) were analyzed across the trials in Figures 4, 5.

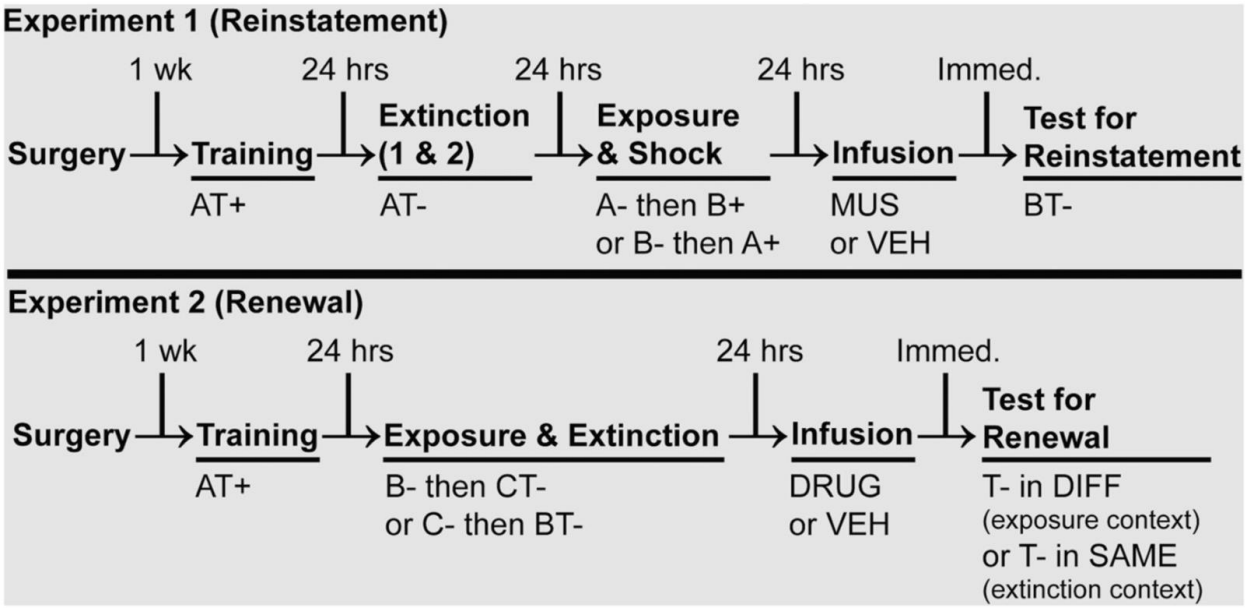

Figure 8. Experimental designs. Experimental designs are read from left ot right. Each phase of behavior is separated by 24 h; however, infusions occurred immediately prior to testing in both Experiments 1 and 2. A, B, C experimental contexts; T, tone CS; +, US; -, no US.

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CHAPTER IV

AMBIGUOUS THREAT SIGNALS DRIVE BNST-DEPENDENT DEFENSE

Introduction

Excessive apprehension about potential future threats, including financial loss, illness, or death, is a defining feature of many anxiety disorders, including generalized anxiety disorder (GAD). Anxiety and trauma-related disorders are widespread and costly (Blanco et al., 2011; Comer et al., 2011; Kinley et al., 2011; Salas-Wright et al., 2014; Stein et al., 2017; Wittchen, 2002), and remain difficult to treat (Colvonen et al., 2017; Costello et al., 2014; Iza et al., 2013; Sinnema et al., 2015). Understanding the neural circuits underlying anxiety is important for refining behavioral and pharmacotherapeutic treatments (Deslauriers et al., 2017; Fanselow and Pennington, 2018, 2017; Graham et al., 2014; LeDoux and Daw, 2018; Nees et al., 2015; Pine and LeDoux, 2017). Several recent studies have demonstrated changes in the activity of the bed nucleus of the stria terminalis (BNST) in individuals with anxiety disorders (Brinkmann et al., 2018, 2017; Buff et al., 2017; Rabellino et al., 2018). However, the conditions that recruit the BNST to aversive learning and memory processes believed to underlie anxiety disorders are still not understood (Avery et al., 2016; Ch'ng et al., 2018; Fox and Shackman, 2017; Goode and Maren, 2017; Gungor and Paré, 2016; Lebow and Chen, 2016; Perusini and Fanselow, 2015; Shackman and Fox, 2016).

Early work on this question revealed that BNST lesions in rats impair defensive behaviors evoked by unconditioned threats (Gewirtz et al., 1998). For example, unconditioned increases in the acoustic startle reflex produced by either intracranial administration of corticotropin releasing factor (CRF) or exposure to bright light require the BNST, whereas fear-

potentiated startle to punctate conditioned stimuli (CSs) do not (Walker et al., 2009). However, the involvement of the BNST in defensive responding is not restricted to unconditioned threat: BNST lesions produce deficits in both freezing and corticosterone release elicited by contextual, but not auditory, CSs after Pavlovian fear conditioning in rats (LeDoux et al., 1988; Sullivan et al., 2004). Based on this work, it has been suggested that the BNST is required to organize behavioral and hormonal responses to sustained threats (whether conditioned or unconditioned). Consistent with this, freezing responses to long-duration auditory conditioned stimuli (CSs) are impaired by BNST lesions (Waddell et al., 2006). Of course, the duration of the behavioral responses in these situations is confounded with the duration of the eliciting stimulus. It has therefore been suggested that sustained defensive *responses* (however they are precipitated) require the BNST (Davis, 2006; Davis et al., 2010; Walker and Davis, 2008; Walker et al., 2009).

Although previous work has focused on the contribution of the BNST to generating defensive responses to sustained threats, another possibility is that the BNST is involved in organizing responses to stimuli that poorly predict *when* an aversive event will occur (Goode and Maren, 2017). For example, punctate auditory CSs that are followed by shock at unpredictable latencies yield sustained freezing responses that are sensitive to BNST manipulations (Daldrup et al., 2016); moreover, freezing in contexts that have been followed by shock at short intervals is not sensitive to BNST lesions (Hammack et al., 2015). This suggests that a crucial parameter that determines the role for the BNST in defensive behavior is neither the duration nor modality of the threat (nor the duration of the elicited defensive response), but rather the information a signal provides about when an aversive event will occur. That is, we propose that the BNST mediates defensive behaviors to temporally uncertain threats, but not stimuli that provide precise

information about when an aversive event will occur. To test this possibility, we examined the role of the BNST using fear conditioning procedures that equated both the duration and modality of the threat CSs, but differed according to the timing of the aversive unconditioned stimulus (US) in relation to the CS. Specifically, we arranged an auditory CS to either precede (forward conditioning) or follow (backward conditioning) a footshock US to directly test contributions of the BNST to defensive responding (freezing in this case) in the presence of temporally predictive or uncertain threat signals. We hypothesized that pharmacological inactivation of the BNST would disrupt fear expression to the backward-trained (but not the forward-trained) CS, because the backward CS poorly predicts when shock will occur. Moreover, we anticipated that the backward CS would increase the activity of BNST neurons and in their afferents implicated in anxiety states.

Results

Reversible inactivation of the BNST attenuates fear to a backward, but not forward, CS.

To examine the role of the BNST in threat uncertainty, we reversibly inactivated the BNST during retrieval of fear to either a forward- ("FW"; certain threat) or backward-trained ("BW"; uncertain threat) CS. A schematic of the behavioral design is shown in Fig. 9. Representative cannula tracts and histological placements are presented in Fig. 17 and 18. Freezing behavior at conditioning is depicted in Fig. 9. A main effect of conditioning trial was observed (repeated measures ANOVA: $F_{6,288} = 37.87$, $P < 0.0001$). No significant main effects or interactions were observed for any of the training/drug assignments across the conditioning trials (ANOVA: F's < 0.70, P's > 0.60). A day later, animals were infused with NBQX, an AMPA receptor antagonist, to reversibly inactivate the BNST; saline ("VEH") infusions served

as a control. Immediately after the infusions, the rats were placed in a novel context and received twelve presentations of the CS (some BW-trained rats received no CS exposure, "No CS") (Fig. 9). Analysis of freezing behavior across the entire session (including baseline) revealed a main effect of trial (repeated measures: $F_{6,264} = 8.06$, $P < 0.0001$), a significant main effect of CS exposure ($F_{2,44} = 25.38$, $P < 0.0001$), and a significant CS-exposure × drug assignment interaction ($F_{2,44} = 3.66$, $P < 0.05$). No other main effects or interactions were detected across the session (ANOVA: F's < 1.6, P's > 0.05). Fisher's PLSD indicated that FW-NBQX exhibited significantly more freezing at test as compared to BW-NBQX ($P < 0.0001$), BW-VEH ($P < 0.05$), No CS-NBQX ($P < 0.0001$), and No CS-VEH ($P < 0.0001$). Additionally, post-hoc comparisons revealed that FW-VEH exhibited significantly more freezing as compared to BW-DRUG ($P < 0.0001$), BW-VEH ($P < 0.01$), No CS-NBQX ($P < 0.0001$), and No CS-VEH ($P < 0.0001$). BW-VEH animals were also found to be significantly different from BW-NBQX ($P < 0.005$), No CS-NBQX ($P < 0.005$), and No CS-VEH ($P < 0.001$) animals.

Given that freezing to the BW CS in the vehicle-treated animals was maximal in the first half of the test, a separate factorial ANOVA was performed on the average percentage of freezing during trials 1-6 (Fig. 9). For average freezing during trials 1-6, a main effect of CS exposure was detected ($F_{2,44} = 18.61$, $P < 0.0001$) as well as a CS-exposure × drug assignment interaction ($F_{2,44} = 3.81$, $P < 0.05$). There was no main effect of drug assignment across all of the groups ($F < 3.00$, $P > 0.05$). Fisher's PLSD revealed significant differences in comparisons of BW-NBQX vs. BW-VEH ($P < 0.0005$), FW-NBQX ($P < 0.0005$), and FW-VEH ($P < 0.0001$). Additionally, significant comparisons were observed for BW-VEH vs. No CS-NBQX ($P < 0.0005$) and No CS-VEH ($P < 0.0005$), as well as FW-DRUG vs. No CS-NBQX ($P < 0.0001$) and No CS-NBQX ($P < 0.0005$). FW-VEH rats were observed to be significantly different from

No CS-NBQX ($P < 0.0001$) and No CS-VEH ($P < 0.0001$). It has been suggested that the BNST mediates sustained but not acute fear responses (Davis et al., 2010). Nonetheless, a growing body of research implicates the BNST in a variety of fear behaviors that may not be limited to long-lasting cues or responses alone (Kiyokawa et al., 2015; Luyck et al., 2017). However, as shown in Fig. 9, BNST inactivation selectively attenuated conditioned freezing to the backward CS, which was less sustained across the course of the test than that to the forward CS.

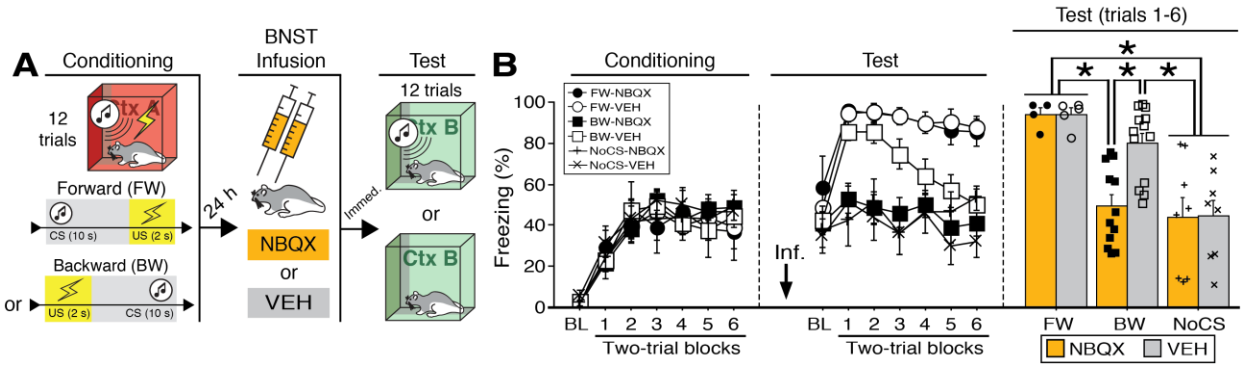

Figure 9. Reversible inactivation of the BNST attenuates conditioned fear expression to a backward, but not forward, CS. (A) Behavioral schematic. **(B)** Freezing at conditioning and testing. For conditioning, left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interval). For testing, center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). Right panel shows mean percentage freezing during the first half of the test (trials 1-6). All data are represented as means ± s.e.m [FW-NBQX ($n = 4$); FW-VEH ($n = 5$); BW-NBQX ($n = 12$); BW-VEH ($n = 13$); NoCS-NBQX ($n = 8$), NoCS-VEH ($n = 8$)]; * = $p < 0.05$.

To establish backward conditioning, we used more conditioning trials than typical of fear conditioning procedures. The failure to affect forward conditioning after BNST inactivation may be due to a ceiling affect in which the high levels of freezing obtained with 12 conditioning trials masked the effects of BNST inactivation. To examine this possibility, we reversibly inactivated

the BNST during retrieval of forward- and backward-conditioned freezing established with five conditioning trials (Fig. 15). The behavioral design for the five-training trial experiment is shown in Fig. 15. The schematic for cannula placements can be found in Fig. 18. Conditioning proceeded normally (Fig. 15), with animals exhibiting increases in freezing across the session (repeated measures ANOVA: $F_{5,115}$ = 12.154, P < 0.0001; no main effects of training/drug assignments and no interaction: F's < 2.3, p's > 0.15). After infusions of NBQX or VEH, animals were tested to the CS in a familiar context (Fig. 15). ANOVA identified a main effect of trial (repeated measures [including baseline freezing]: $F_{5,105}$ = 22.118, P < 0.0001), a main effect of training procedure ($F_{1,21}$ = 15.930, P = 0.0007), and a trial × training procedure interaction (repeated measures: $F_{5,105}$ = 12.346, P < 0.0001). A separate ANOVA performed on trials 1-5 (excluding baseline; Fig. 15) indicated a significant main effect of training procedure ($F_{1,21}$ = 21.585, P = 0.0001; there was no main effect of drug and no interaction: F's < 0.15, P's > 0.7). Thus, BNST inactivation did not alter responding to the FW or BW CS when trained using five trials; however, fear expression was significantly lower in BW animals, and did not appear to increase after baseline (suggesting a possible floor effect). Together, these experiments indicate that twelve conditioning trials were required to establish freezing to the backward CS, which was sensitive to BNST inactivation, whereas forward-conditioned freezing obtained after either 5 or 12 trials was not. In sum, these data indicate that the BNST is required for the expression of conditioned freezing to a backward, but not forward, CS.

The differential effect of BNST inactivation on freezing to a forward or backward CS suggests that the BNST regulates defensive behavior to stimuli that poorly signal US onset. One index that might reveal differences in the ability of the CS to predict the US, is the US-evoked response itself. Footshock USs elicit an unconditioned response (UR), that includes vocalization,

autonomic adjustments, and burst of locomotor activity (Bali and Jaggi, 2015; Fanselow, 1994). To better understand the mechanisms of forward and backward conditioning on behavior, we examined shock-evoked activity bursts (Kunwar et al., 2015; Zelikowsky et al., 2018) during the conditioning session in a separate cohort of animals (a separate cohort was analyzed in order to compare equal numbers of FW- and BW-trained animals) (Fig. 16). Fear conditioning resulted in robust freezing in both groups of animals (Fig. 16). An ANOVA revealed a significant main effect of trial ($F_{6,180} = 67.02$, $P < 0.0001$) with no differences between levels of freezing in FW or BW animals (no other main effect and no interaction: F's < 1.80, P's > 0.15). In contrast, shock-induced activity differed in FW and BW animals (Fig. 16). All animals exhibited a reliable decrease in shock-induced activity across the conditioning session ($F_{5,150} = 9.59$, $P < 0.0001$) and the rate of decline in activity was similar in the two groups (no trial × group interaction: $F < 1.70$, $P > 0.09$), but the overall level of shock-induced activity was significantly higher in BW animals (main effect of group: $F_{1,30} = 9.59$, $P < 0.05$). For comparison, two-tailed unpaired *t*-test of mean levels of activity during the 5-min baseline revealed no significant difference between FW and BW rats (Fig. 16; $t < 0.30$; $P > 0.75$). Hence, USs that were not signaled by a forward CS evoked greater activity bursts than those that were. Collectively, these data suggest that temporal uncertainty is an important factor in recruiting the BNST to fear.

Temporary inactivation of the BNST does not eliminate fear to a forward CS that is paired with a US of variable intensity.

To determine whether the BNST is involved in other conditioning procedures imbued with outcome uncertainty, we examined whether freezing to a forward CS that is paired with a US of variable intensity is also BNST-dependent. In this case, rats received forward fear

conditioning with either a fixed ("FIXED") or variable ("VARIABLE") US intensity (Fig. 10). A schematic of the behavioral design is shown in Fig. 10; cannula placements are illustrated in Fig. 18. During conditioning (Fig. 10), a repeated measures ANOVA revealed a main effect of trial ($F_{6,168}$ = 68.15, P < 0.0001), with no main effect of drug or training assignment, and no interactions (F's < 1.50, P's > 0.20).

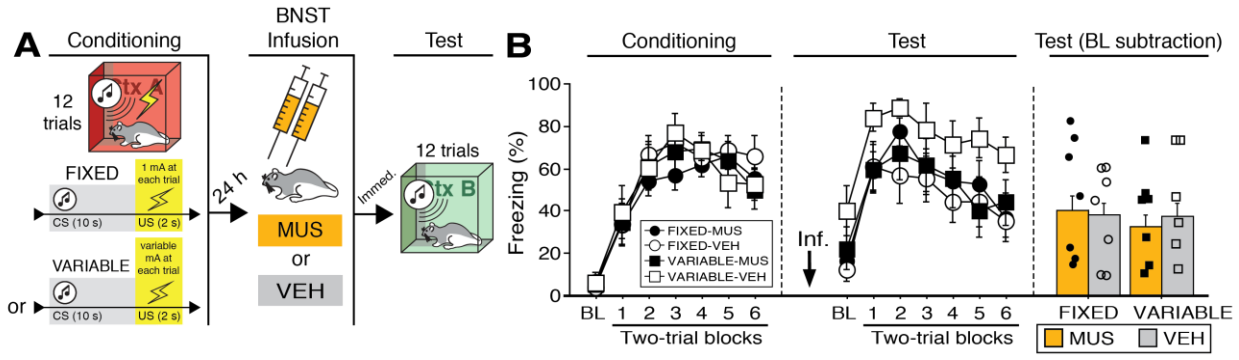

Figure 10. Temporary inactivation of the BNST does not prevent conditioned fear expression to a forward CS paired with a US of fixed or variable intensity. (A) Behavioral schematic. **(B)** Freezing at conditioning and testing. For conditioning, left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 58-sec interval). For testing, center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). Right panel shows mean percentage freezing across all test trials (after BL), with BL levels of freezing subtracted from these values. All data are represented as means ± s.e.m [FIXED-MUS (n = 8); FIXED-VEH (n = 7); VARIABLE-MUS (n = 8); VARIABLE-VEH (n = 7)].

Twenty-four hours later and immediately before a test to the CS in a novel context (Fig. 10), the animals were infused with muscimol ("MUS") to reversibly inactivate the BNST; saline vehicle ("VEH") infusions served as a control. During retrieval testing, there was a main effect of trial ($F_{6,156}$ = 24.31, P < 0.0001), however no other main effects or interactions were detected (F's < 2.70, P's > 0.15) suggesting that BNST inactivation did not affect the expression of freezing to a forward CS paired with a variable intensity US. Although the mean for baseline

freezing was highest in VARIABLE-VEH animals, factorial ANOVA of baseline freezing did not result in any main effects or interactions (F's < 3.60, P's > 0.05). To equate for differences in pre-CS freezing in the experimental groups, baseline responding was subtracted from CS-elicited freezing (Fig. 10). This results in no significant main effects or interactions in the factorial ANOVA (F's < 0.15, P's > 0.70). Thus, while BNST inactivation trended towards reducing generalized contextual freezing, drug infusions did not significantly impact freezing to the temporally predictable CS, regardless of US intensity.

Backward CSs selectively increase Fos expression in the ventral BNST.

To further examine the role of the BNST in the expression of fear to a backward CS, we quantified Fos expression in multiple subregions of the BNST following the presentation of either a forward or backward CS during a shock-free retrieval test (Fig. 12). The behavioral design is summarized in Fig. 11. Four experimental groups were compared: rats trained and tested to a forward CS ("FW"), rats trained and tested to a backward CS ("BW"), rats trained to a forward or backward CS but not receiving a CS at test ("No-CS"), and animals that were trained but not tested ("No Test"). Conditioning (Fig. 11) was similar to previous experiments (main effect of trial: $F_{6,258} = 77.346$, $P < 0.0001$; no other main effects or interactions: F's < 1.30, P's > 0.30). Freezing during the retrieval test is shown in Fig. 11. A repeated measures ANOVA revealed a main effect of trial ($F_{6,216} = 15.54$, $P < 0.0001$), test group ($F_{2,36} = 11.42$, $P < 0.0001$), and a test group × trial interaction ($F_{6,216} = 3.65$, $P < 0.0001$) at test (includes baseline). For these data, post-hoc analyses revealed that FW ($P < 0.0001$) and BW ($P < 0.005$) rats exhibited significantly higher levels of freezing behavior than No-CS rats. Similarly, a factorial ANOVA of mean responding across trials 1-12 revealed a main effect of group ($F_{2,36} = 13.76$, $P < 0.0001$),

with post-hoc comparisons indicating significant differences between FW vs. BW ($P < 0.05$) and FW vs. No CS rats ($P < 0.0001$), as well as BW vs. No-CS animals ($P < 0.005$).

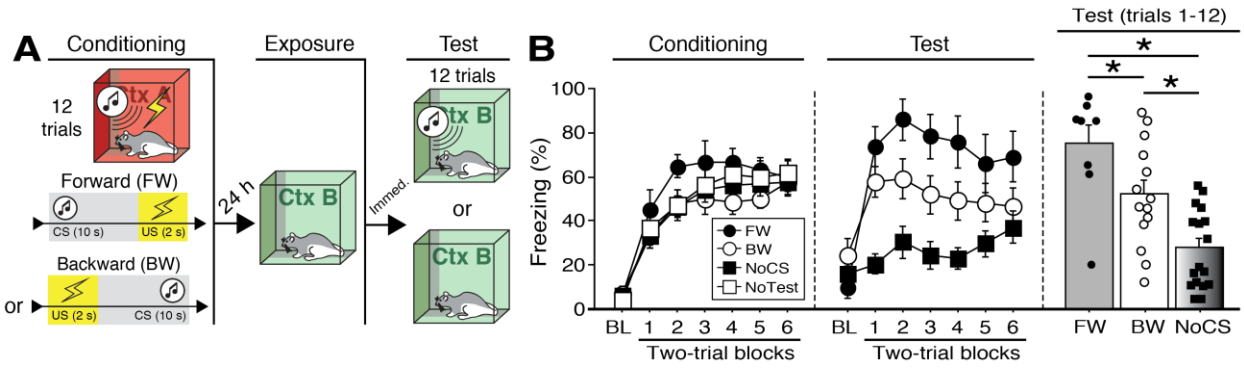

Figure 11. CS-evoked freezing in rats utilized for Fos analyses. (A) Behavioral schematic. **(B)** Freezing at conditioning and testing. For conditioning, left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interval). For testing, center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). Right panel shows mean percentage freezing after BL. Animals were sacrificed for Fos analyses 90 min after trial 1. All data are represented as means ± s.e.m [FW (n = 8); BW (n = 14); NoCS (n = 17); NoTest (n = 8)]; * = $p < 0.05$.

Ninety minutes after the retrieval test, the animals were sacrificed for Fos immunohistochemistry. Fos-positive nuclei were counted in three BNST subregions (Fig. 12): "ovBNST" (Fos counts confined to the oval nucleus of the BNST), "am(dorsal)BNST" (counts in an area containing the dorsal region of the anteromedial BNST), and "al/fu/am(ventral)BNST" (Fos counts in a region containing the BNST's anterolateral, fusiform, and anteromedial [ventral] nuclei) [refer to (Swanson, 2003)]. The average number of Fos-positive nuclei for each of these regions in each group are shown in Fig. 12. Factorial ANOVA detected a main effect of group for Fos in al/fu/am(ventral)BNST ($F_{3,43} = 11.41$, $P < 0.0001$). Fisher's PLSD identified BW rats as exhibiting significantly higher levels of Fos in this region as compared to FW ($P < 0.0005$),

No CS (P < 0.001), and No Test (P < 0.0001) animals. Additionally, No CS animals were significantly higher as compared to No-Test rats (P < 0.05). For Fos in am(dorsal)BNST, we did not detect a significant group effect (factorial ANOVA: F < 2.60, P > 0.07). Factorial ANOVA of the Fos data within ovBNST revealed a main effect of group ($F_{3,43}$ = 3.26, P < 0.05). Post-hoc analyses further identified significant differences, such that No-Test rats exhibited significantly higher Fos levels vs. FW (P < 0.05) and No-CS (P < 0.01) rats. Finally, these data indicate exposure to a BW CS is associated with enhanced Fos expression in the BNST, particularly in its ventral regions; an outcome which is consistent with our inactivation studies. Moreover, exposure to the temporally predictive FW CS (which elicited the highest levels of fear) was associated with low levels of Fos.

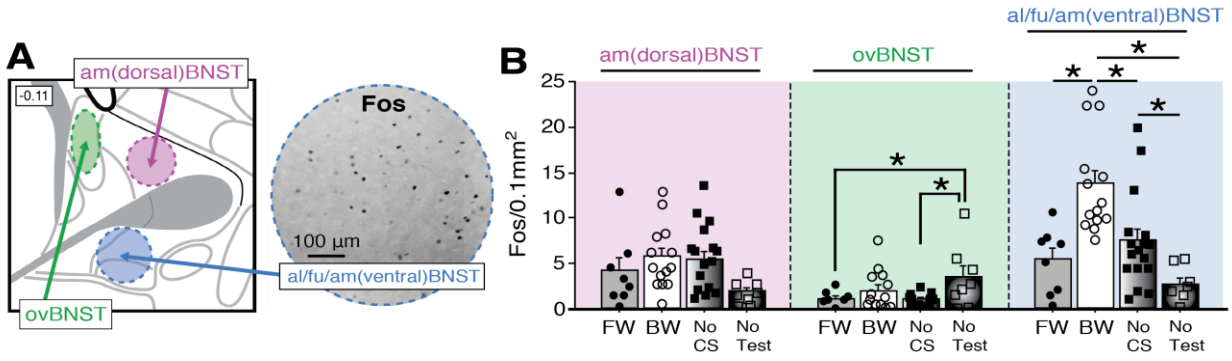

Figure 12. Fos expression in the BNST following exposure to a temporally predictable or uncertain CS. (A) Schematic depicting regions counted within the BNST (left panel). Right panel shows example of Fos expression in the al/fu/am(ventral)BNST. **(B)** Mean Fos-positive cells per 0.1 mm^2 for each of the quantified regions. All data are represented as means ± s.e.m [FW (n = 8); BW (n = 14); NoCS (n = 17); NoTest (n = 8)]; * = p < 0.05.

Backward CSs selectively increase Fos expression in mPFC afferents of the BNST.

The BNST receives input from many areas involved in the regulation of fear (Fox and Shackman, 2017). Therefore, we used a functional tracing procedure to quantify Fos expression in neurons targeting the BNST. Specifically, rats were injected with retrograde tracer (CTb-488)

into the BNST (prior to behavior), and we examined levels of activity in regions known to target the BNST, including the infralimbic and prelimbic regions of the prefrontal cortex, the basolateral amygdala, and the ventral hippocampus (Fig. 13). The behavioral data for these animals corresponds to the data shown in Fig. 11.

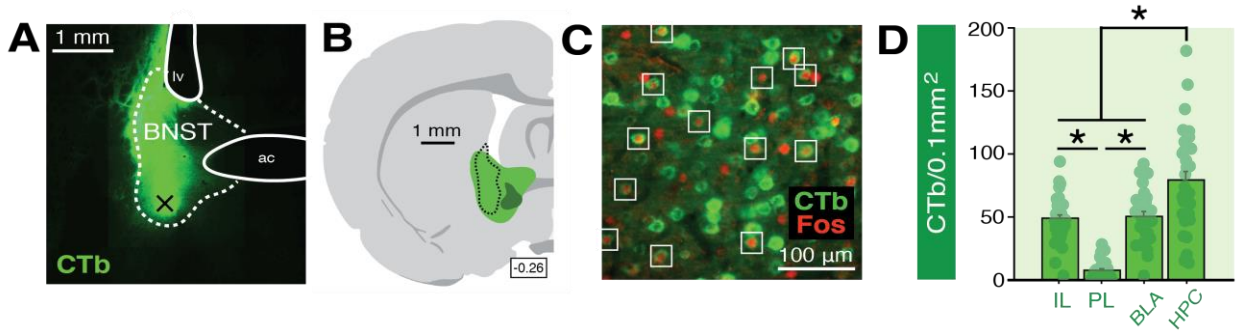

Figure 13. Functional tracing in afferents targeting the BNST. (A) Coronal section (10×) showing representative fluorescence of CTb infusion (green) into the BNST (dotted outline; "ac" = anterior commissure, "lv" = lateral ventricle). Black "X" denotes approximate lowest point of the infusion (as an example of how injection sites are documented in Fig. S5). **(B)** Coronal schematic (-0.26 mm from bregma) showing the approximate largest (green) and smallest (dark green) areas of CTb spread in the BNST for animals included in the analyses; the black dotted outline represents the extent of spread of CTb in the BNST in the image shown in panel **A**. **(C)** Example CTb-positive (green) and Fos-positive (red; nuclei) cells in a coronal section (40 µm) of the IL; open white squares denote double-labeled cells. **(D)** Mean number of BNST-targeting CTb-positive cells (per 0.1 mm^2) for each of the quantified regions (shows FW, BW, and NoTest animals corresponding to Fig. 6). All data are represented as means ± s.e.m (for each region, n = 30); * = $p < 0.05$.

A representative image of CTb infusion into the BNST is shown in Fig. 13. An illustration of the largest and smallest CTb spread of injection included in the analyses is shown in Fig. 13. Approximate microinjection sites for CTb for all animals are shown in Fig. 19. Representative Fos and CTb co-labeling is shown in Fig. 13. Average CTb counts in each region of interest are depicted in Fig. 13. Collapsing across behavioral groups, factorial ANOVA of CTb-positive counts revealed a significant main effect of region ($F_{3,116}$ = 42.34, $P < 0.0001$).

Post-hoc comparisons indicated that the number of CTb-positive cells in the HPC were significantly higher than all other regions (P < 0.0001, per comparison), with IL and BLA exhibiting significantly greater CTb counts as compared to PL (P < 0.0001, per comparison). CTb counts were similar across all behavioral conditions (factorial ANOVA, split by region; no main effects of group or interactions: F's < 0.5, P's > 0.75). These data indicate extensive connectivity of the PFC, BLA, and HPC with the BNST.

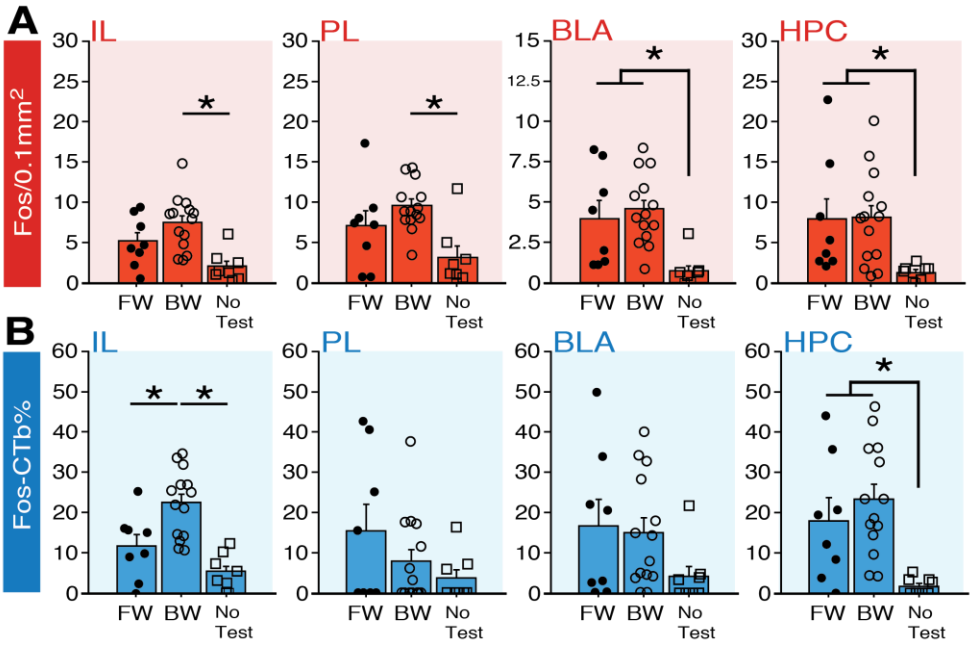

Figure 14. Fos expression in BNST-targeting cells of prefrontal cortex, amygdala, and hippocampus following exposure to a temporally predictable or uncertain CS. (A) Mean number of Fos-positive cells (per 0.1 mm^2) for each of the quantified regions. **(B)** Mean percentage of Fos-positive and CTb-positive cells divided by the total number of CTb-positive cells for each region. All data are represented as means ± s.e.m [FW (n = 8); BW (n = 14); NoTest (n = 8)]; * = p < 0.05.

As shown in Fig. 14, the number of Fos-positive nuclei in the IL, PL, HPC, and BLA differed among the behavioral groups. Factorial ANOVA of Fos counts in IL revealed a main effect of group ($F_{2,27}$ = 8.55, P < 0.005). Post-hoc comparisons for IL revealed significant

differences between BW vs. No Test rats ($P < 0.0005$) and FW vs. No-Test rats ($P < 0.05$). In PL, a main effect of group was also identified (factorial ANOVA: $F_{2,27} = 6.79$, $P < 0.005$). Post-hoc analyses indicated that BW rats exhibited significantly more Fos in PL as compared to No-Test animals ($P < 0.001$). For BLA, a main effect of group was detected (factorial ANOVA: $F_{2,27} = 8.10$, $P < 0.005$). Post-hoc analyses revealed that FW and BW rats (which did not significantly differ) exhibited significantly more Fos in BLA as compared to No Test rats ($P < 0.01$, FW vs. No Test; $P < 0.001$, BW vs. No Test). In HPC, factorial ANOVA revealed a significant main effect of group ($F_{2,27} = 4.28$, $P < 0.05$). Post-hoc comparisons indicated that FW and BW rats (again, which did not significantly differ) had significantly more Fos expression in HPC as compared to No Test animals ($P < 0.05$ for FW vs. No Test and BW vs. No Test). These data indicate that conditioned freezing to forward or backward CSs increased the number of Fos-positive neurons in the PFC, BLA, and HPC.

To quantify the fraction of BNST-projecting neurons within the PFC, BLA, and HPC that were activated by a forward or backward CS, we calculated a ratio of Fos-positive to CTb-positive nuclei in each afferent region ("Fos-CTb%"; Fig. 14). Interestingly, within the IL, factorial ANOVA revealed a significant main effect of group ($F_{2,27} = 14.22$, $P < 0.0001$). Post-hoc comparisons revealed significant differences between BW and FW rats ($P < 0.005$) as well as BW and No Test animals ($P < 0.0001$). Thus, we observed increased activation of BNST-targeting cells of IL when comparing BW and FW rats. For PL, which had low numbers of CTb-positive cells, no significant main effects of group were detected for Fos-CTb% (factorial ANOVA: $F < 1.90$, $P > 0.17$). Although it exhibited greater numbers of CTb-positive cells, no group effects were observed for Fos-CTb% in the BLA (factorial ANOVA: $F < 2.0$, $P > 0.15$). For HPC, a significant main effect in the factorial ANOVA was detected ($F_{2,27} = 7.82$, $P < 0.01$).

Post-hoc comparisons indicated significant differences between FW vs. No-Test rats ($P < 0.05$) and BW vs. No Test rats ($P < 0.0005$). Accordingly, these data indicate that conditioned freezing (in both FW and BW rats) is associated with increased activity in BNST-targeting cells of HPC. Thus, IL projections to the BNST may account for differences in levels of BNST activity, and/or overall levels of freezing to the differentially trained CSs.

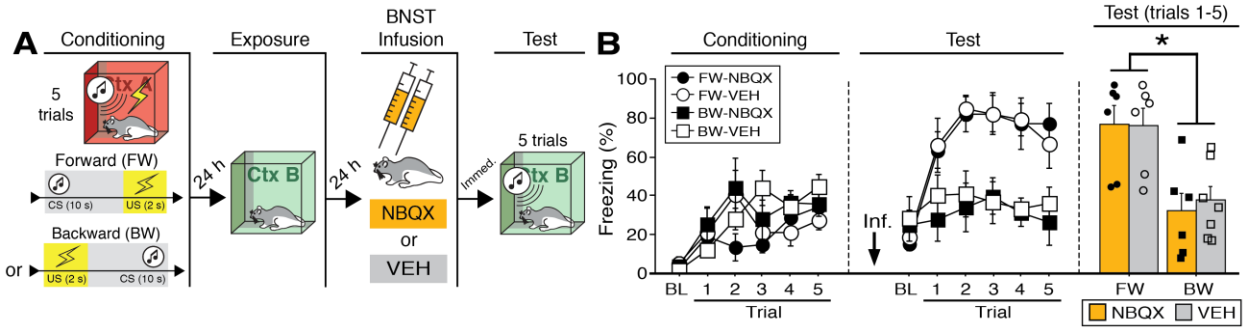

Figure 15. Effects of BNST inactivation on freezing to a forward vs. backward CS trained with five trials. (A) Behavioral schematic. **(B)** Freezing at conditioning and testing. For conditioning, left panel depicts mean percentage freezing during the 3-min baseline (BL) and across each conditioning block (each block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interval). For testing, center panel shows mean percentage freezing at the 3-min baseline (BL) and across each test block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). Right panel shows mean percentage freezing after baseline (trials 1-5). All data are represented as means ± s.e.m [FW-NBQX (n = 6); FW-VEH (n = 6); BW-NBQX (n = 6); BW-VEH (n = 7)]; * = $p < 0.05$.

Given that Fos-CTb% levels were elevated in IL in BW animals, we examined whether pharmacological activation of the IL with the $GABA_A$ antagonist, picrotoxin ("PTX"), increases the number of Fos-positive nuclei within the BNST (Fig. 20). Picrotoxin infusion into the IL administration resulted in significantly more Fos expression in am(dorsal)BNST as compared to vehicle (t_{11} = 3.156, $P < 0.01$). A similar effect was demonstrated for Fos in al/fu/am(ventral)BNST as compared to vehicle (t_{11} = 2.465, $P < 0.05$). No significant difference

was observed for Fos in the ovBNST following drug or vehicle infusion into the IL ($t < 2.1$, $P > 0.05$).

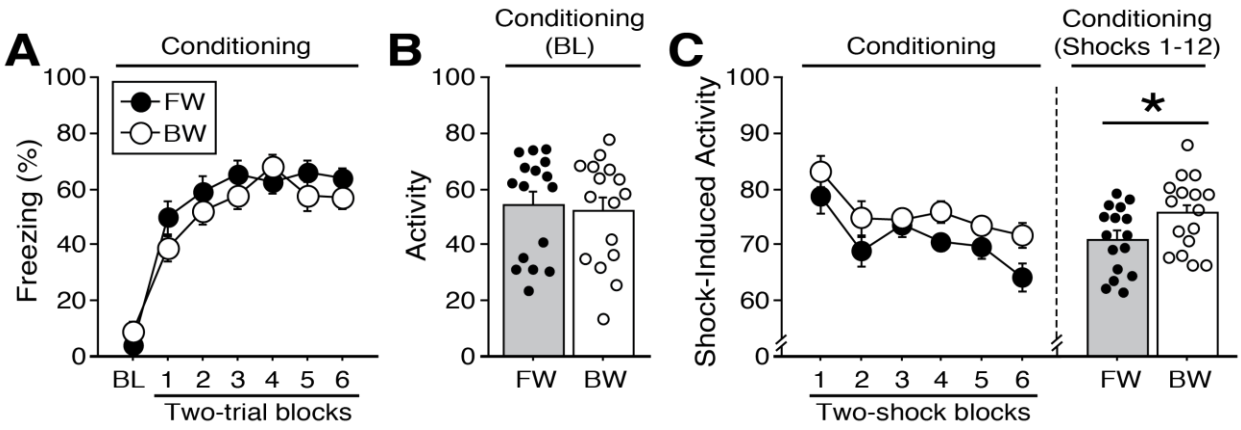

Figure 16. **Shock-induced activity during conditioning to a forward vs. backward CS. (A)** Mean percentage freezing at baseline (BL; 5-min) and across conditioning blocks (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 58-sec interval). **(B)** Mean activity values across the 5-min BL (no shock present). **(C)** Left panel shows mean shock-induced activity during conditioning (averaged into two-shock blocks). Right panel shows mean shock-induced activity across all trials. All data are represented as means ± s.e.m [FW (n = 16); BW (n = 16)]; * = $p < 0.05$.

Given this relationship, and in light of IL's role as an essential regulator of fear inhibition (Marek et al., 2018; Milad and Quirk, 2002), we also examined whether pharmacological inhibition of the IL disrupted fear expression to a backward (but not forward) CS (Fig. 21). The behavioral schematic for this experiment is documented in Fig. 21. A representative image of cannula placements in IL is shown in Fig. 22, with placements documented for all rats in Fig. 22. Conditioning data for these animals are shown in Fig. 21. A repeated measures ANOVA revealed a main effect of trial ($F_{6,156}$ = 83.050, $P < 0.0001$), but with no main effect of drug or training assignment and no group interactions (F's < 1.5, P's > 0.30). Animals were infused with muscimol ("MUS") or vehicle ("VEH") immediately before a test to the CS in a novel context

Fig. 21. A repeated measures ANOVA performed on the data from the retrieval test revealed a significant main effect of trial ($F_{6,156}$ = 10.514, P < 0.0001), and a significant training group × trial interaction ($F_{6,156}$ = 2.398, P < 0.05); no other significant main effects or interactions were detected across the entire test (F's < 3.8, P's > 0.05). That said, factorial ANOVA of freezing during post-baseline freezing indicated a main effect of drug ($F_{1,26}$ = 4.291, P < 0.05), but with no main effect of training assignment and no interaction (F's < 1.1, p's > 0.30). These data suggest that IL is likely involved in regulating BNST activity, but it may not drive BNST-dependent freezing *per se*; IL inactivation increased freezing across the test to both the FW and BW CS. In total, the Fos and CTb data indicate distinct patterns of activity in afferent projections to the BNST during the expression of conditioned freezing to the FW or BW CS, particularly with regards to the PFC, suggesting some possible role of cortical regulation of the BNST during fear expression.

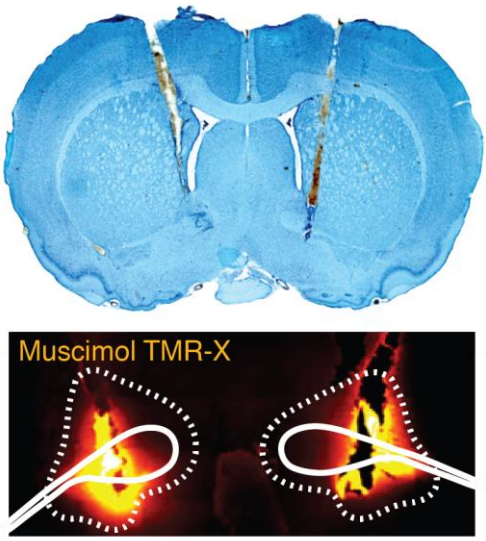

Figure 17. Representative bilateral cannula placements in the BNST. Photomicrograph (10×) of a thionin-stained coronal section depicting representative cannula tracts in the BNST (top panel). Fluorescent image (gold filter) of a coronal section (10×) showing spread of drug in BNST (BNST outlined in dotted line) (bottom panel).

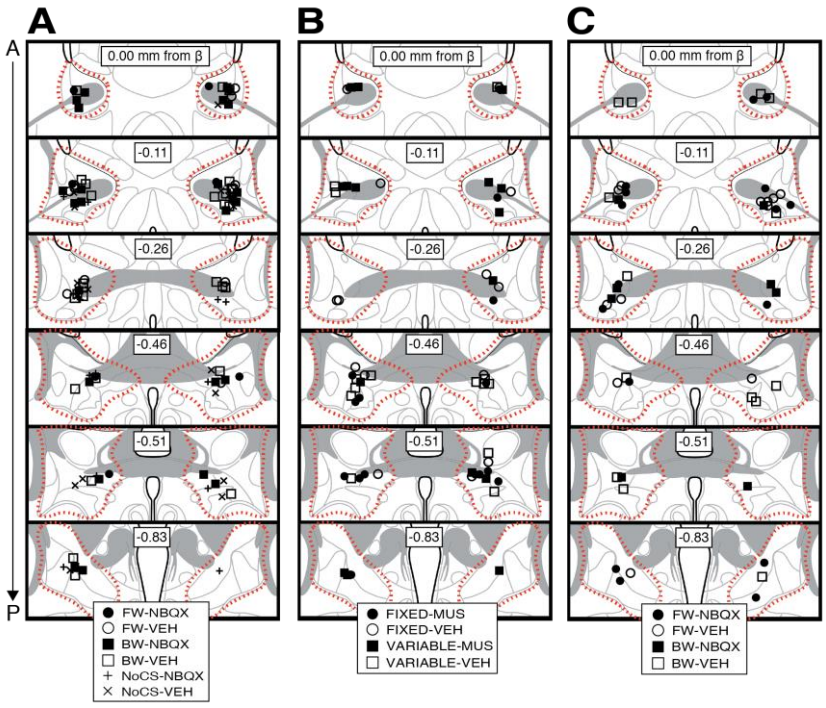

Figure 18. Bilateral cannula placements for each experiment involving BNST microinfusions. (A) Schematic depicting cannula placements for Fig. 1. **(B)** Schematic depicting cannula placements for Fig. 2. **(C)** Schematic depicting cannula placements for Fig. S1. For all schematics, symbols (split by each group) correspond to injector tips (approximate borders of BNST are shown by red dotted outline).

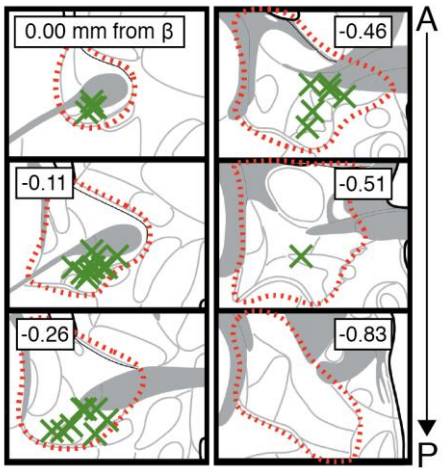

Figure 19. CTb injection sites in BNST. Most ventral and centermost (approximate) sites of unilateral microinjection of CTb (green X's) for all animals shown in Fig. 5 (red outline approximates borders of BNST).

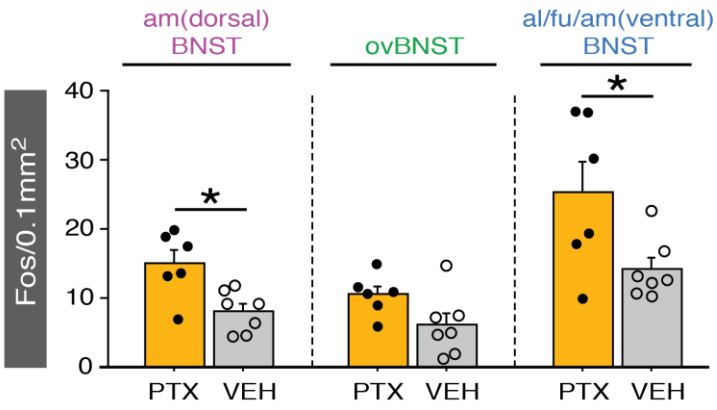

Figure 20. Fos expression in the BNST following pharmacological activation of IL. Mean levels of Fos expression in regions of the BNST (identical to regions shown in Fig. 4) following intra-IL infusion of picrotoxin (microinfusions were administered 72 hrs after the final test shown in Fig. S7; rats for the analyses were a randomly selected subset of animals from the IL-inactivation experiment [includes BW- and FW-trained rats; only rats with cannula placements in IL are included] and were sacrificed 95 min after the infusions). All data are represented as means ± s.e.m [PTX (n = 6), VEH (n = 7)]; * = $p < 0.05$.

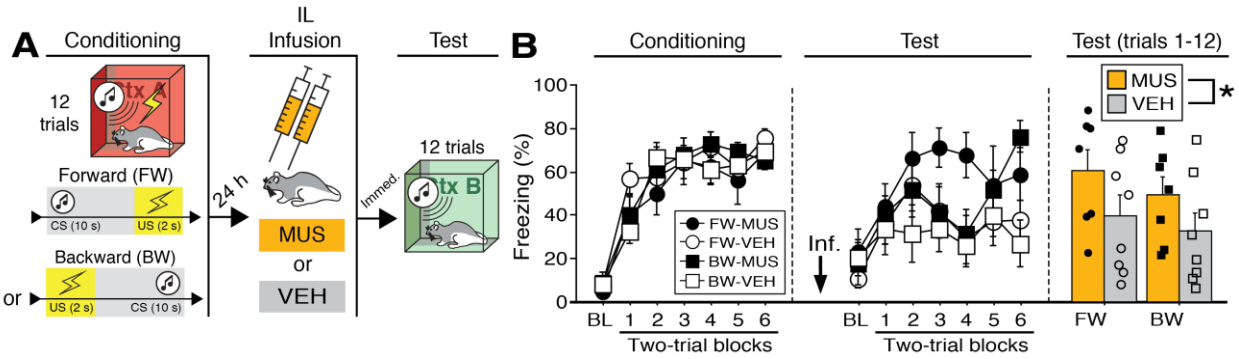

Figure 21. Effects of IL inactivation on fear expression to a forward and backward CS. (A) Behavioral schematic. **(B)** Freezing at conditioning and testing. For conditioning, left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interval). For testing, center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). Right panel shows mean percentage freezing after BL (trials 1-12). All data are represented as means ± s.e.m [FW-MUS (n = 7); FW-VEH (n = 8); BW-MUS (n = 7); BW-VEH (n = 8)]; * = $p < 0.05$.

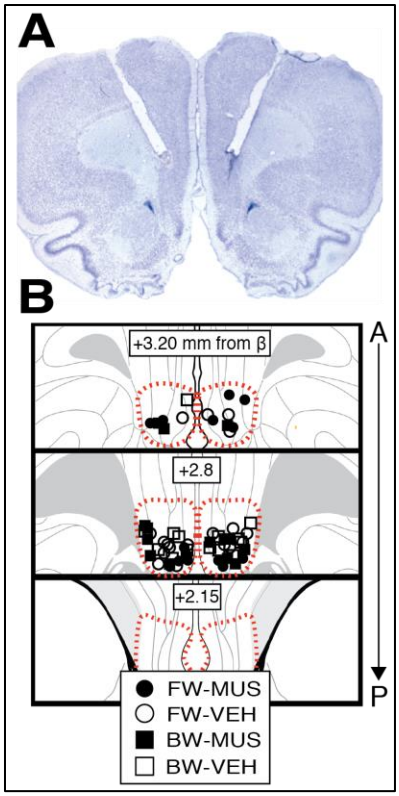

Figure 22. Bilateral cannula placements for experiments involving IL microinfusions. (A) Photomicrograph (10×) of a thionin-stained coronal section depicting representative cannula tracts in the IL. **(B)** Schematic depicting cannula placements for Fig. S7. Symbols (split by each group) correspond to injector tips (approximate borders of IL are shown by red dotted outline).

Discussion

We demonstrate for the first time that expression of conditioned freezing to a temporally ambiguous threat, such as to an excitatory backward CS, is associated with increased Fos expression in the BNST and that reversible inactivation of the BNST attenuates these defensive behaviors. In contrast, temporary inactivation of the BNST did not attenuate conditioned fear to a temporally certain forward CS—whether trained with five or twelve trials, and even when that CS signaled an unpredictable intensity of the US. Interestingly, these effects occurred despite the result that freezing levels persisted for longer in the presence of the forward CS than with the backward CS. Additionally, shock-induced activity during conditioning trials were higher overall

in rats trained to the backward CS as compared to the forward CS. Finally, CS-evoked fear retrieval broadly coincided with increased Fos expression in the BLA and HPC, as well as with the activation of BNST-projecting cells of HPC. However, backward CS-exposed rats had greater Fos expression (as compared to No Test animals) in the PFC. Backward CS-exposed rats also had greater Fos expression in BNST-targeting cells of IL. Although pharmacological activation of the IL increased Fos in the BNST, pharmacological inactivation of the IL did not disrupt fear expression and actually increased the expression of conditioned fear in the later portion of the test. Collectively, these data reveal a critical role for the BNST in processing temporally uncertain threats.

Unpredictability comes in many forms (Bennett et al., 2018; Davies and Craske, 2015; McNally et al., 2011; Schroijen et al., 2016) and uncertainty regarding the onset of aversive events has been linked to anxiety (Bennett et al., 2018). For example, work in humans (Shankman et al., 2011) and animals (Amadi et al., 2017) suggests that unpredictable timing of aversive stimuli is a key contributor to anxious symptoms, and there now exists evidence of activity in the BNST during cases in which uncertainty of US onset is a factor during conditioning (Alvarez et al., 2015; Klumpers et al., 2017). Likewise, backward conditioning may elicit a defensive state during which the animal is uncertain of when the US will occur (though the animal clearly expects the US to happen, given the increases in freezing). Anticipating uncertain threats is also a feature of other BNST-dependent behaviors, including contextual fear expression and context fear-dependent relapse (Davis and Walker, 2014; Goode et al., 2015; Hammack et al., 2015; Luyten et al., 2011; Sullivan et al., 2004; Waddell et al., 2006; Zimmerman and Maren, 2011). Interestingly, it has been suggested that fear to a backward CS relies on associations between the CS and the conditioning context (Chang et al., 2003), which

more strongly predicts the US. Thus, it is possible that the BNST mediates fear expression to a backward CS by disrupting expression of the memory of the context-US association. In either event, the BNST appears to be recruited by stimuli, whether cues or contexts, that are poor predictors of when aversive USs will occur. Furthermore, we also observed significant differences in the intra-shock activity of animals during conditioning to the FW and BW CS. Perhaps these differences contribute to the recruitment of the BNST to aversive learning. There are data pointing towards the BNST as mediating the negative effects of unpredictable shock and learned helplessness (Hammack et al., 2004). Additionally, there is evidence to suggest that uncertainty of shock onset is more aversive to humans than uncertainty of whether the negative event will occur at all (Bennett et al., 2018).

Another possibility is that the backward conditioning procedures that we have used establishes a forward conditioned association between the CS and the US occurring after the one minute "trace" interval (Burman et al., 2014; Marchand et al., 2004; Raybuck and Lattal, 2014; Tipps et al., 2014). Although there has been considerable work on the neural mechanisms of trace conditioning (Raybuck and Lattal, 2014), a role for the BNST in this form of learning has not yet been established. Because trace conditioning degrades the temporal predictability of the CS, it might be expected to also requires the BNST.

An important feature of this work is that we demonstrate that the BNST mediates conditioned freezing to short-duration cues, particularly when those cues poorly predict shock onset. Prior work has suggested that cue duration regulates recruitment of the BNST to freezing behaviors, such that the BNST is necessary only for long-lasting cues or contexts (Hammack et al., 2015; Waddell et al., 2006). However, emergent data have indicated a role for the BNST in threat responses to relatively brief stimuli (Brinkmann et al., 2018; Kiyokawa et al., 2015; Luyck

et al., 2017). Thus, these data suggest that cue duration is not necessarily a determinant of BNST involvement.

Also in the present work, conditioned freezing to the backward CS was generally lower and shorter lived than to that of the forward CS. This might suggest that the BNST mediates weak (but not strong) freezing responses. However, we and others have shown that relatively high levels of fear expression can be reduced by BNST lesions or inactivation (Goode et al., 2015; Hammack et al., 2015). On the other hand, the null effects of BNST inactivation on fear expression to the forward CS doesn't appear to be entirely due to a ceiling effect, as BNST inactivation also failed to diminish freezing to a forward CS conditioned with five trials. These results are similar to studies that have observed null effects of BNST lesions on forward CS-elicited fear using fewer trials than the current study (Goode et al., 2015; LeDoux et al., 1988; Sullivan et al., 2004). Furthermore, we observed no effect of BNST inactivation on forward CS fear expression, despite higher and longer-lasting levels of freezing across the tests. Thus, rather than suggest the BNST mediates sustained fear responding, we argue that the BNST mediates fear to cues that signal uncertain threat, and that this uncertainty can in some cases induce sustained fear responses (Goode and Maren, 2017).

Others have shown increases in immediate early gene expression in the BNST in response to conditioned contexts (Campeau et al., 1997; Lemos et al., 2010). Metabolic activity in the BNST has also been shown to be active during exposure to a conditioned context (Luyten et al., 2012). If the backward CS functions similarly to a conditioned context, then one might expect Fos activity to be elevated in the BNST of animals exposed to the backward CS. Indeed, we observed significantly more Fos expression in the ventral regions of the BNST as compared to forward CS rats or rats not undergoing retrieval. The backward CS-elicited Fos expression

was subregion-dependent, insofar as backward and forward rats did not differ in overall levels of Fos in am(dorsal)BNST or ovBNST. Others have shown elevated Fos expression in regions in or near al/fu/am(ventral)BNST following BNST-related fear activation and other stressors (Sterrenburg et al., 2012; Verma et al., 2018). Interestingly, we found significantly more Fos labeling in the ovBNST of animals not undergoing testing as compared to forward CS or no retrieval rats (but not as compared to backward CS-exposed rats). Note that the no-retrieval rats exhibited low levels of Fos in the other subregions of the BNST. While it is unknown what cell-types are Fos-positive in our study, these findings are nevertheless interesting because prior work has suggested that the cells of the ovBNST are important for anxiogenic behaviors (Kim et al., 2013). Additionally, others have reported higher levels of Fos in ovBNST following various stressors (Day et al., 2004; Kormos et al., 2016). We find it unlikely that our No-Test animals (housed in their homecages during testing) were more anxious than our FW rats, which exhibited high levels of conditioned freezing. It is important to consider that the BNST, and particularly ovBNST, is a site of circadian rhythm-related activity (Amir et al., 2006, 2004; Fuchs et al., 1996; Yamazaki et al., 1998). Thus, it is possible that the levels of Fos observed in the No-Test animals reflects circadian activity that is not being disrupted by behavioral testing.

Elevated Fos activity in the BLA of animals exposed to an aversive CS (as compared to animals not receiving CS retrieval) is consistent with prior reports observing fear retrieval-induced Fos in the BLA (Hall et al., 2001; Izumi et al., 2011). It is interesting that levels of Fos were similar between forward and backward CS animals in our study. While forward and backward CS-exposed animals differed in overall levels of freezing at retrieval, it is unclear whether this difference is substantial enough to result in differential Fos expression in BLA. While the number of trials in the Fos experiments was chosen so as to match the reversible

inactivation experiments in this study (and rats were sacrificed so as to maximize Fos levels following the onset of CS exposure), it is possible that the length of the test begins to engage some fear inhibition mechanisms (given the window of time in which Fos can express). Perhaps this possibility is reflected in the overall levels of Fos (Herry et al., 2008). Similar to BLA, increased levels of Fos were observed in the HPC of animals tested to the fear CS (as compared to animals not undergoing retrieval). Others have reported HPC activation in fear-retrieving animals and in animals exposed to a familiar context (as compared to homecage controls) (Jin and Maren, 2015; Wang et al., 2016). Although forward and backward CS-retrieving rats did not significantly differ in overall levels of Fos in IL or PL, BW rats did exhibit higher levels of Fos as compared to No Test rats for both IL and PL (FW and No Test animals did not differ). This suggests perhaps some enhanced activity in the PFC during fear retrieval to the backward CS—an outcome which may be similar to reports showing elevated Fos in the PFC of rats exposed to a conditioned context (Lemos et al., 2010). Relatedly, exposure to predator odor has been shown to increase ΔFosB immunoreactivity in regions of IL and PL (Mackenzie et al., 2010)—behavioral responding to predator odor has been shown to rely on the BNST (Breitfeld et al., 2015; Fendt et al., 2003).

The amygdala, PFC, and hippocampus have strong connections with the BNST (Canteras and Swanson, 1992; deCampo and Fudge, 2013; Dong et al., 2001; Glangetas et al., 2017; McDonald et al., 1999; Reichard et al., 2017; Reynolds and Zahm, 2005; Torrisi et al., 2015; Vertes, 2004; Weller and Smith, 1982), though the contributions of these afferents to BNST-dependent fear behaviors are not well understood. Here we show that forward and backward CSs increase Fos expression in BNST-targeting cells of BLA and HPC to a similar degree, and the number of BNST-targeting cells in the HPC was significantly higher as compared to No-Test

controls. HPC projections to the BNST are thought to regulate hypothalamic-pituitary-adrenal (HPA)-axis activity (Crestani et al., 2013; Forray and Gysling, 2004; Zhu et al., 2001). Perhaps their activation by forward and backward CSs relates to the retrieval of memories of the familiar test context (because rats were exposed to the context before the test), rather than fear expression *per se* (an outcome that may also be true for the BLA→BNST neurons, given the low levels of baseline fear).

Interestingly, our data suggests some possible regulatory mechanisms of PFC on BNST-dependent behavior. Specifically, Fos activity was higher in BNST-targeting cells of IL of backward CS rats as compared to the other groups. Similarly, pharmacological activation of the IL was associated with increased Fos in the BNST. IL projections to the BNST are known to be glutamatergic (Crowley et al., 2016; Glangetas et al., 2017), and activity in IL appears to regulate BNST-dependent anxiety-like behaviors (Glangetas et al., 2017) and may be involved in BNST-dependent reinstatement of drug seeking (Reisiger et al., 2014). Furthermore, ethanol-induced hyperexcitability of IL neurons has been shown to coincide with enhanced activity in ventral regions of the BNST (Pleil et al., 2015). Additionally, humans with damage to the vmPFC were shown to exhibit weakened BNST activity at rest as compared to healthy humans (Motzkin et al., 2015). Thus, it is possible IL is involved this form of fear expression—however, IL has an important role in fear suppression and extinction (Bloodgood et al., 2018; Marek et al., 2018). As such, and given the lower levels of fear in the BW rats, it is possible IL is acting on the BNST to minimize defensive responding in the presence of the uncertain CS. This may be acting throughout the course of exposure, given the lower levels of freezing in backward vs. forward rats during the entire test. However, the levels of Fos in CTb-positive cells of IL may also reflect fear inhibitory mechanisms that may be engaged towards the end of the test. Likewise,

inactivation of the IL appeared to prop up fear responding (particularly at the end of the test) in both FW and BW rats. Possible functional dissociations of IL and PL with regards to fear inhibition and activation (respectively) have been examined extensively (Sun et al., 2018). Perhaps related to these possibilities, we observed that IL appears to exhibit considerably more inputs to BNST than PL (which is consistent with prior tracing studies). Activity in PL has been shown to be important for contextual fear (Corcoran and Quirk, 2007; Zelikowsky et al., 2013)—a form of fear expression that presumably relies on the BNST; nonetheless, our data suggest that BNST-projecting PL neurons may not be selectively driving fear for the backward fear animals given the similar levels across groups.

To conclude, we demonstrate a novel role for the BNST in defensive responding to a backward CS, suggesting that outcome uncertainty in the form of ambiguous timing of negative events is an important overarching factor in BNST-dependent behavior. We note that the current data does not address other potential contributors to conditioned fear-related BNST activity such as the CeA (Asok et al., 2018; Li et al., 2012), dorsal raphe nucleus (DRN) (Marcinkiewcz et al., 2016), and orbitofrontal cortex (OFC) (Fox et al., 2010). However, the current data suggest some coordination of activity in BNST through the PFC, BLA, and HPC. In total, our results bring new insight on how the BNST may be involved in conditioned behaviors, particularly behaviors that are elicited in the presence of uncertain threat.

Materials and methods

Subjects

All experiments utilized adult (200-240 g upon arrival; n = 247, before exclusions) male Long-Evans rats (Envigo; Indianapolis, IN). Rats were housed in a climate-controlled vivarium

and kept on a fixed light/dark cycle (lights on starting at 7:00 AM and off at 9:00 PM; experiments took place during the light phase of the cycle). Rats were individually housed in clear plastic cages (with sawdust bedding; changed weekly) on a rotating cage rack. Group assignments for behavioral testing was randomized for cage position on the racks. Animals had access to standard rodent chow and water *ad libitum*. Animals were handled by the experimenter(s) (~30 sec/day) for five consecutive days prior to the start of any surgeries or behavior. All procedures were in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Apparatuses

All behavioral testing occurred within distinct rooms in the laboratory. Each behavioral room housed eight identical rodent conditioning chambers (30 cm × 24 cm × 21 cm; MED Associates, Inc.). Each chamber was housed in a larger, external sound-attenuating cabinet. Rear walls, ceilings, and the front doors of the testing chambers were made of Plexiglas, while their sidewalls were comprised of aluminum. Grid floors of the chambers were comprised of nineteen stainless steel bars (4 mm in diameter), and spaced 1.5 cm apart (center to center). The grid floors were attached to an electric shock source and a solid-state grid scrambler for delivery of the US (MED Associates, Inc.). A speaker was attached to each rodent chamber for delivery of the auditory CS. As needed for each context, the chambers were equipped with 15 W house lights, and small fans were embedded in the cabinets (providing background noise of ~70 dB). An aluminum pan was inserted beneath the grid floor to collect animal waste. A small camera was attached to the top of the cabinet for video monitoring of behavior.

Measurements of freezing were performed using an automated system (Maren, 1998). Specifically, each behavioral testing chamber rested on a load-cell platform that was sensitive to cage displacement due to the animal's movements. During behavioral testing, load-cell activity values (ranging from -10 to +10 V) were collected and digitized at 5 Hz using Threshold Activity Software (MED Associates, Inc.). Offline conversions of the activity values were performed to generate absolute values ranging from 0 to 100; low values indicate minimal cage displacement, which coincide with freezing behaviors in the chambers. Accordingly, freezing bouts were defined as absolute values of ≤10 for 1 s or more. The extent of freezing was then analyzed as percentages of time as described for each experiment. Shock reactivity was analyzed by directly reporting the absolute values generated by the Threshold Activity Software (i.e., larger values indicated more movement in the cage).

Unique contexts (A and B) were generated as needed for the behavioral procedures. Chamber assignments were unique to each context and group assignments were counterbalanced across test chambers when possible. For each experiment, context A was assigned to one of the behavioral rooms, and B the other. For context A, the test chamber was wiped down with an acetic acid solution (3%) and a small amount was poured in the pans beneath the grid floors. The cage lights were turned on, while the chamber fans were turned off. The cupboard doors were closed. The behavioral room was illuminated with dim red light. Animals were transported to and from the context using white plastic transport boxes. For context B, an ammonium hydroxide solution (1%) was used to wipe down and scent the chambers. Thin black plastic sheets were placed over the grid floors. The cage lights were turned off, while the chamber fans were turned on. Cupboard doors remained open. The behavioral room was illuminated with white light (red

room lights were turned off). Rats were transported to and from the context using black plastic transport boxes that included a layer of clean sawdust bedding.

Surgeries

For animals receiving intracranial microinfusions into the BNST [similar to prior reports (Acca et al., 2017; Goode et al., 2015; Nagaya et al., 2015; Zimmerman and Maren, 2011)], rats were transported from the vivarium to a surgical suite and deeply anesthetized using isoflurane (5% for induction, 1-2% for maintenance). Rats were then secured in a stereotaxic frame (Kopf Instruments). Hair on top of the rodent's head was shaved, povidone-iodine was applied, and a small incision was made in the tissue to expose the top of the skull. Holes were drilled into the skull to attach small jeweler's screws. Bregma and lambda of the skull were aligned on an even plane, additional small holes were drilled into the skull, and bilateral stainless-steel guide cannulas (26 gauge, 8 mm from the bottom of their plastic pedestals; Small Parts) were slowly inserted into the BNST at the following coordinates: -0.15 mm posterior to bregma, ±2.65 mm lateral to the midline, and -5.85 mm dorsal to dura (guide cannulas were angled at 10° with their needles directed at the midline). Dental cement was used to build a headcap and to secure the cannulas to the screws. Stainless steel obturators (33 gauge, 9 mm; Small Parts) were inserted into the guide cannulas. Animals were allotted at least one week to recover prior to the onset of behavioral training. The final data reflects rats with bilateral cannula tips terminating within the borders of the BNST. For animals receiving intracranial microinfusions into the IL [similar to prior reports (Giustino et al., 2017; Marek et al., 2018)], animals were prepped for surgery as described above. Bilateral stainless-steel guide cannulas (identical to above) were slowly inserted into the IL at the following coordinates: +2.7 mm anterior to bregma, ±3.0 mm lateral to the

midline, and -4.9 mm dorsal to dura (guide cannulas were angled at 30° with their needles directed at the midline). A headcap was secured as described above, and animals recovered for one week prior to behavioral training. The final data reflects rats with bilateral cannula tips terminating within the borders of the IL.

For rats injected with cholera toxin subunit B (CTb) conjugated with Alexa Fluor-488 (CTb-488; ThermoFisher Scientific) in the BNST, rats were again transported to the surgical suite, anesthetized, and prepped for surgery as described above. Bregma and lambda were aligned on an even plane. A single small hole was drilled into the skull to allow for the insertion of the injector. Rats received unilateral CTb-488 infusions into either the left or right hemisphere (group assignments were randomized for sites of CTb-488 infusion). For use in the injector, borosilicate capillaries were inserted into a micropipette puller (Narishige International USA, Inc.), and pulled to provide fine injection tips. Injection tips were backfilled with mineral oil and secured in the injector; CTb-488 was then drawn up into the injector immediately before use. When ready, the injection pipette was lowered to the following coordinates in the BNST: -0.15 mm posterior to bregma, ±2.65 mm lateral to the midline, and -6.50 mm dorsal to dura (the pipette was angled at 10° with the tip directed at the midline). CTb-488 (5.0 mg/µl; total volume of 0.25 µl) was microinfused into the brain using a Nanoject II auto-nanoliter injector (Drummond Scientific Co.) secured to the arms of the stereotaxic frame. For the infusion process, 50 nl (25 nl/s) of CTb-488 was infused once per min for 5 min to achieve 0.25 µl of total infusion (the injection needle was left in the brain for 5 additional minutes to allow for diffusion of CTb-488). Following the infusion procedures, the incision was stitched up were returned to their homecages. Animals recovered for ~10 days following the CTb infusions.

Intracranial infusions

Twice before the start of behavioral testing, animals were acclimated to the process of intracranial infusions. This process involved transporting the animals from the vivarium to a separate room used for drug infusions. Animals were transported in 5-gallon buckets containing a layer of sawdust bedding. Experimenter(s) removed the obturators and replaced them with clean ones. On the day of infusions, animals (in squads of four to eight rats; representing all drug/behavioral groups as possible) were transported to the laboratory, obturators were removed, and injectors were inserted into the guides. 9 mm stainless steel injectors (33 gauge, Small Parts; extending 1 mm beyond the end of the guide cannula, once inserted) were connected to polyethylene tubing (PE-20; Braintree Scientific), with the other end of tubing connected to gastight 10 µl syringes (Hamilton, Co.). The syringes were mounted to an infusion pump (KD Scientific, Inc.). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) was used to reversibly inactivate the BNST (Adami et al., 2017; Davis and Walker, 2014; Goode et al., 2015; Zimmerman and Maren, 2011). NBQX disodium salt hydrate (Sigma Life Sciences) was dissolved in saline to a concentration of 10.0 µg/µl ("NBQX"); physiological saline served as the vehicle ("VEH" for all experiments). Additionally, γ-aminobutyric acid $(GABA)_A$ receptor agonist muscimol was used to reversibly inactivate the BNST (Bangasser et al., 2005; Breitfeld et al., 2015; Buffalari and See, 2011; Fendt et al., 2003; Goode et al., 2015; Markham et al., 2009; Pina et al., 2015; Sajdyk et al., 2008; Xu et al., 2012). Muscimol (Sigma-Aldrich) was dissolved in physiological saline to a concentration of 0.1 µg/µl ("MUS"); physiological saline served as the vehicle ("VEH"). For the representative image showing drug spread in the BNST, muscimol TMR-X conjugate (Thermo Fisher Scientific) was dissolved in physiological saline to

a concentration of 0.1 μg/μl and used for infusions. Also, the GABA receptor antagonist picrotoxin was used to temporally activate the IL (Chang and Maren, 2011; Marek et al., 2018). Picrotoxin (Tocris Bioscience) was dissolved in physiological saline to a concentration of 0.2 μg/μl ("PTX"); physiological saline served as the vehicle ("VEH"). Muscimol was used to reversibly activate the IL (Marek et al., 2018). Muscimol (Sigma-Aldrich) was dissolved in physiological saline to a concentration of 0.1 μg/μl ("MUS"); physiological saline served as the vehicle ("VEH"). For all of the aforementioned experiments, drug or vehicle was drawn up into the injectors (immediately prior to the infusions), and a total volume of 0.275 μl of drug or vehicle was infused at a rate of 0.275 μl/min, injectors were left in the cannulas for 1 min following the infusions to allow for diffusion. Once the injectors were removed, clean obturators were inserted into the guides.

Behavioral procedures and exclusions

General overviews of each behavioral experiment are provided in the figures. The discrete conditioned stimulus (CS) for all experiments was an auditory tone (80 dB, 2 kHz, 10 sec). A 1.0 mA, 2 sec footshock served as the unconditioned stimulus (US) for all experiments, except for the uncertain shock intensity experiment in which some of the animals received variable shock intensities (but equal duration) at training.

FW/BW BNST inactivation (twelve training trials)

In a 3 × 2 design, animals (n = 64, prior to exclusions) were randomly assigned to receive a CS at test [either a forward ("FW")- or backward ("BW")-trained CS] or no CS at test [animals were trained to a BW CS; "NoCS"], and NBQX ("NBQX") or vehicle infusions ("VEH"). Of

these rats, seven were excluded due to off-target cannulas, three additional rats were excluded due to illness, and four more were excluded due to a technical error during infusions that resulted in no drug or vehicle to be infused (fourteen total exclusions). This resulted in the following (final) group numbers (shown in data/figures): FW-NBQX (n = 4); FW-VEH (n = 5); BW-NBQX (n = 12); BW-VEH (n = 13); NoCS-NBQX (n = 8), NoCS-VEH (n = 8). At the start of behavior, animals (in squads of eight rats) were transported from the vivarium to context A to begin fear conditioning. We alternated running FW and BW squads at conditioning. Drug and vehicle assignments were counterbalanced for position in the chambers. For FW conditioning, animals received a period of 5 min to acclimate to the context, then began the onset of twelve CS-then-US pairings (CS offset immediately preceded US onset). Exactly 1 min following the offset of each CS, onset of the next CS began. Following the final CS-US pairing, the animals remained in the chamber for 1 min before being returned to their homecages (the entire conditioning session consisted of 19 min total; for both FW and BW conditioning). For BW conditioning, all aspects of conditioning were identical to the FW training, however, the order of the CS and US was reversed. That is, after a 5-min baseline in the context, the first US began, and at its offset, CS onset occurred. 60 sec intertrial intervals separated the offset of the CS with the onset of the following CS. After conditioning, rats were returned to their homecages.

24 hrs after conditioning, animals (in squads of four) were infused with DRUG or VEH into the BNST immediately before being placed in context B. For rats receiving the CS at test, and after 5 min of acclimation to the context, FW and BW animals (intermixed in each squad) received twelve presentations of the CS in the absence of the US. Exactly 1 min following the offset of each CS, onset of the next CS began, and rats remained in the chambers for 1 min following the final CS (19 min session, in total). For rats not receiving the CS at test, the animals

remained in context B for 19 min without the CS or US. We alternated running squads that received the CS and those that did not after the infusions. Following the test, animals were returned to their homecages.

FW/BW BNST inactivation (five training trials)

In a 2 × 2 design, animals (n = 28, prior to exclusions) were randomly assigned to receive forward ("FW") or backward ("BW") conditioning, and NBQX ("NBQX") or vehicle infusions ("VEH"). Of these rats, three were excluded due to off-target cannulas. This resulted in the following (final) group numbers (shown in data/figures): FW-NBQX (n = 6); FW-VEH (n = 6); BW-NBQX (n = 6); BW-VEH (n = 7). At the start of behavior, rats (in squads of seven) were transported from the vivarium and placed in context A. We alternated squads that received FW or BW conditioning. For FW rats, they were allotted 3 min of acclimation to the context prior to the onset of five CS-then-US pairings. Each CS was separated by 1 min (offset to onset), and US onset occurred immediately following the offset of each CS. Rats remained in the chamber for 1 min following the final trial (530 sec total conditioning session). After a 3-min baseline (530 sec session total), BW rats received five US-then-CS trials (CS onset occurred at US offset), with each CS separated by 1 min (offset to onset). Rats were returned to their homecages after conditioning. 24 hrs after conditioning, animals were placed in context B in the absence of the CS or US for 530 sec. After this acclimation session, rats were again returned to their homecages.

24 hrs later, animals underwent NBQX or VEH infusions (identical to above). FW and BW animals were intermixed in each squad. Immediately following the infusions, animals were placed in context B for a test to the CS. The test (530 sec in total) consisted of a 3-min baseline

period followed by five CS-alone presentations (separated by 1 min intervals, offset to onset of the CS). Rats were returned to their homecages following the test session.

FIXED/VARIABLE shock w/ BNST inactivation

In a 2 × 2 design, rats (n = 31, prior to exclusions) were randomly assigned to receive forward conditioning with consistent ("FIXED") or variable magnitudes of the US ("VARIABLE"), and muscimol ("MUS") or vehicle infusions ("VEH"). For FIXED animals, the magnitude of the shock at conditioning was consistently set to 1 mA. For VARIABLE animals, animals experienced the following levels of shock (in mA, and in this exact order) at each conditioning trial (mean = 1 mA): 0.5, 1.8, 0.4, 1.6, 1.4, 0.3, 0.5, 1.8, 0.4, 1.6, 1.4, 0.3. Of these rats, one rat was excluded due to off-target cannulas. This resulted in the following (final) group numbers (shown in data/figures): FIXED-MUS (n = 8); FIXED-VEH (n = 7); VARIABLE-MUS (n = 8); VARIABLE-VEH (n = 7). At the start of behavior, animals (in squads of seven to eight) were transported to context A for conditioning (squads alternated between FIXED and VARIABLE paradigms). We alternated running squads of FIXED and VARIABLE animals. For both FIXED and VARIABLE animals, rats were allotted 5 min to acclimate to the context before the onset of CS-US pairings. For all squads, the CS preceded the US, and US onset coincided with CS offset. Animals experienced twelve training trials, with 1 min separating the offset of the CS with the onset of the following CS. Rats remained in the chambers for 1 min following the final CS, with the entire conditioning session lasting 19 min. Rats were returned to their homecages following conditioning.

24 hrs later, rats (in squads of seven to eight) underwent intracranial infusions of DRUG or VEH into the BNST before being placed in context B. Rats experienced a 5-min baseline

period before the onset of twelve CS-alone presentations, separated by 1 min intervals. Rats remained in the chambers for 1 min following the final CS, with the entire test session lasting 19 min. Rats were returned to their homecages following the test.

FW/BW Fos-CTb

Animals (n = 60, before exclusions) were randomly assigned to receive a forward ("FW")- or backward ("BW")-trained CS at testing, or no CS retrieval at test ("NoCS"). Note that the NoCS group consists of animals that were trained to either a FW or BW CS. Additionally, a group of BW-trained animals remained in their homecages ("NoTest") during the final test and were sacrificed alongside the other groups. Twelve rats were excluded for either excessive and off-target infusion of CTb-488 outside the borders of the BNST or by lacking CTb-488 spread into its ventral nuclei (since the ventral regions of the BNST were observed to have significant elevations of Fos expression, we restricted our analyses to include only animals that had CTb-488 in its ventral regions). One additional rat was excluded due to a technical issue that resulted in the loss of tissue at the level of the prefrontal cortex. This resulted in the following (final) group numbers (shown in data/figures): FW (n = 8); BW (n = 14); NoCS [n = 17 (BW-trained: n = 9; FW-trained: n = 8)]; NoTest (n = 8). At the start of behavioral training, rats (in squads of six to eight; with all groups intermixed) were transported to the laboratory and placed in context B to acclimate for 5 min (no CS or US). Animals were then returned to their homecages. Later that day, animals were transported to context A to undergo twelve trials of FW or BW fear conditioning, which was identical to the other aforementioned procedures. We alternated FW and BW conditioning squads. After conditioning, rats were returned to their

homecages. 24 hrs after conditioning, all rats were exposed to context B for 20 min in the absence of the CS or US.

24 hrs later and in squads of three to four, FW, BW, and NoCS rats were transported to context B to receive CS or no CS retrieval. Squads alternated between CS and no CS retrieval. For rats undergoing CS retrieval, FW and BW animals (intermixed in each squad) experienced CS trials as identical to the other aforementioned procedures that used twelve test trials. Rats were perfused 90 min following the first CS of the test, in groups of three to four. For NoCS rats (with FW- and BW-trained animals intermixed), animals were exposed to context B in the absence of the CS or US. NoCS rats were perfused 95 min after being placed in the test context, in groups of three to four. NoTest rats (one or two at a time) were perfused alongside groups of FW, BW, and NoCS rats. Rats were returned to their homecages after testing and prior to the perfusions.

FW/BW intra-shock reactivity

Rats (n = 32, no exclusions) were randomly assigned to receive forward ("FW") or backward ("BW") conditioning. No rats were excluded from this experiment (no infusions occurred; n = 16, per group); only data from conditioning is shown. At the start of behavior, animals (in squads of eight) were transported to context A for either FW or BW conditioning. Parameters for FW and BW conditioning were identical to procedures for our other experiments involving twelve FW or BW trials. We alternated FW and BW squads. Rats were returned to their homecages following training.

FW/BW IL inactivation

In a 2 x 2 design, rats (n = 32, prior to exclusions) were randomly assigned to undergo forward ("FW") or backward ("BW") conditioning, and muscimol ("DRUG") or vehicle infusions ("VEH"). Of these rats, two rats were excluded due to off-target cannula. This resulted in the following (final) group numbers (shown in data/figures): FW-MUS (*n* = 7); FW-VEH (*n* = 8); BW-MUS (*n* = 7); BW-VEH (*n* = 8). At the start of behavior, animals (in squads of eight) were transported to context A for either FW or BW conditioning. Parameters for FW and BW conditioning were identical to procedures for our other experiments involving twelve FW or BW trials. We alternated FW and BW squads. 24 hrs after conditioning, animals (in squads of eight) were infused with DRUG or VEH into the IL immediately before being placed in context B. Parameters for testing in context B was identical to other experiments involving twelve test trials. Rats were returned to their homecages following the test.

IL activation w/ BNST Fos

At the conclusion of the FW/BW IL inactivation experiment, a random subset of these rats (n = 14, prior to exclusions) were randomly assigned to receive intra-IL infusions of picrotoxin ("PTX") or vehicle ("VEH") 95 min before being sacrificed (rats were returned to their homecages prior to being sacrificed). Of these rats, one rat was excluded due to a technical issue that resulted in the loss of tissue at the level of the BNST (this rat is included in the behavioral data described above). This resulted in the following (final) group numbers (shown in data/figures): PTX (*n* = 6); VEH (*n* = 7).

Histological procedures

At the conclusion of behavioral testing, cannula-implanted animals were overdosed using sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml, i.p.). Transcardial perfusions were then performed using chilled physiological saline followed by 10% formalin. Brains were extracted and stored in 10% formalin for 24 hr at 4° C; brains were switched to a 30% sucrose-formalin solution for three or more days (at 4° C) before sectioning. Brains were flash frozen with crushed dry ice and coronal sections (40 μm) containing the BNST were collected using a cryostat (Leica Microsystems) at -20° C. The tissue was wet-mounted to gelatin-subbed microscope slides and stained with 0.25% thionin prior to adding glass coverslips secured with mounting medium (Permount, Sigma). To further examine the spread of drug, a subset of animals was infused (identical to the aforementioned infusion parameters) with fluorescent muscimol (1.0 μg/μl; EverFluor TMR-X conjugate, Setareh Biotech) before being sacrificed (these animals were not perfused) and having brains dissected (40 μm; brains were stored in 30% sucrose solution at 4° C until sectioning). These tissues were wet-mounted to slides and aqueous mounting medium (Fluoromount; Sigma-Aldrich) was used to secure glass coverslips.

For CTb-injected animals, post-behavior perfusions mirrored the aforementioned procedures. For sectioning, coronal sections (which included regions of the prefrontal cortex, BNST, basolateral amygdala, and ventral hippocampus) were collected into well plates containing phosphate-buffered saline (1× PBS) and stored in the dark at 4° C until immunohistochemistry could be performed. For localization of the CTb-injection site, separate sections at the level of the BNST were wet-mounted and coverslipped using Fluoromount mounting medium.

Immunohistochemistry

Immunohistochemistry for Fos was performed on free-floating brain tissue; similar to prior reports (Jin and Maren, 2015; Marek et al., 2018; Orsini et al., 2011; Wang et al., 2016). For sections containing the BNST, Fos was stained using the following procedures [all steps were performed at room temperature (~20° C) on a shaker, unless stated otherwise; rinses were brief (~20 sec)]. The tissues were first rinsed in 1× tris-buffered saline (TBS; 7.4 pH), and then incubated in 0.3% H_2O_2 (in TBS) for 15 min, followed by rinses (×3) in TBS. Slices were transferred to primary antibody [rabbit anti-c-fos, 1:10,000 in 1× TBS containing Tween 20 (TBST); Millipore] and incubated overnight. Sections were then rinsed (×3) in TBS before incubating in secondary antibody for 1 hr (biotinylated goat anti-rabbit, 1:1,000 in TBST; Jackson Immunoresearch). Sections were rinsed (×3) again in TBS. The slices were transferred to wells containing avidin biotin complex (ABC, 1:1,000 in TBST; Vector Labs) for 45 min. The tissues were again rinsed (×3) in TBS. Tissue was transferred to wells containing 3,3′ diaminobenzidine [(DAB) 5% stock, 1:200], nickel ammonium sulfate (5% stock, 1:10), and 30% H_2O_2 (1:2,000) in TBS for 10 min to generate purple/black nuclear products. After another rinsing (×3) in TBS, the tissues were subsequently wet-mounted to slides and secured with coverslips using Permount mounting medium.

For sections containing the prefrontal cortex, amygdala, and hippocampus, Fos was stained using the following procedures [unless stated otherwise, each step occurred at room temperature (~20° C) on a shaker (and away from excess light)]. First, the tissues were rinsed (10 min; ×2) in 1× TBS, followed by a 10 min wash in 1× TBST. The tissues were incubated in 10% normal donkey serum (NDS; in TBST) for 1 hr. The slices were then rinsed (5 min; ×2) in TBST. Sections were transferred to primary antibody [goat anti-c-fos, 1:2,000 in 3% NDS (in

TBST); Santa Cruz Biotechnology] and incubated on a rotator in the dark for 72 hr at 4° C. The tissues were rinsed (10 min; ×3) in TBST before incubating in secondary antibody [biotinylated donkey anti-goat, 1:200 in 3% NDS (in TBST); Santa Cruz Biotechnology] for 2 hr. Slices were then rinsed (10 min; ×3) in TBST. Sections were transferred to wells containing streptavidin (Alexa-Fluor 594-conjugate, 1:500 in 3% NDS (in TBST); Thermo Fisher Scientific] for 1 hr. Tissues were rinsed in (10 min; ×3) in TBS, before being wet-mounted to slides and secured with coverslips using Fluoromount mounting medium.

Image analyses

All imaging and counting procedures (for all regions) were performed with the experimenter(s) blind to the group assignments of the animals. For thionin-stained coronal tissue, photomicrographs of cannula in the BNST were generated (10× magnification) using a Leica microscope (MZFLIII) and Leica Firecam software. For animals infused with fluorescent muscimol into the BNST, infusion sites were imaged (10× magnification) using a Zeiss microscope and Axio Imager 2 software (Zen Pro 2012). For CTb-injected animals, BNST images were generated (10× magnification) using the same Zeiss microscope and software.

For Fos analyses in BNST, brightfield images of BNST [in BNST regions ranging from approximately -0.00 to -0.50 mm posterior to bregma of the skull, and from both left and right hemispheres (randomized for site of CTb-injection)] were generated (10× magnification) using a Zeiss microscope and Axio Imager 2 software (Zen Pro 2012). ImageJ software (National Institutes of Health) was used to count cells. Counts were confined to the following areas of interest: (1) “ovBNST” [an area of 0.217 mm × 0.558 mm (oval); targeting the oval nucleus of the BNST], (2) “am(dorsal)BNST” [0.372 mm^2 (circle); targeting the dorsal and medial

subregions of the anterior BNST, including the anteromedial area of the BNST (dorsal to the anterior commissure)], and (3) "am(ventral)/fu/alBNST" [0.434 mm^2 (circle); targeting the ventral regions (ventral to the anterior commissure) of the anterior BNST, which includes the ventral portion of the anteromedial area, the anterolateral area, and the fusiform nucleus of the BNST] (Swanson, 2003). For each of these regions, three to six images were quantified and averaged for each animal (Fos levels were standardized to 0.1 mm^2).

For Fos-CTb analyses in the prefrontal cortex, amygdala, and hippocampus, images (10× magnification) of Fos and CTb expression were generated using a Zeiss microscope and Axio Imager 2 software (Zen Pro 2012), and counts were analyzed using ImageJ. Images were only generated for the hemisphere injected with CTb for these areas. Counts were confined to the following areas of interest: (1) "IL" [an area of 0.805 mm × 0.217 mm (rectangle); targeting deep and superficial layers of the infralimbic cortex], (2) "PL" [1.115 mm × 0.217 mm (rectangle); targeting deep and superficial layers of the prelimbic cortex], (3) "BLA" [0.434 mm^2 (circle); targeting the basolateral amygdala, and (4) "HPC" [0.496 mm × 0.217 mm (rectangle); targeting the ventral subiculum but may include some CA1 cells of the ventral hippocampus (Swanson, 2003). For each of these regions, three to six images were quantified and averaged for each animal (all counts were normalized to 0.1 mm^2).

Statistics

All data were submitted to repeated or factorial analysis of variance (ANOVA) or two-tailed *t*-tests as appropriate and as described for each experiment. Fisher's protected least significant difference (PLSD) test was used for *post hoc* comparisons of group means following a significant omnibus *F* ratio in the ANOVA (α was set at 0.05). No statistical methods were used

to predetermine group sizes (group sizes were selected based on prior work and what is common for the field). Data distributions were assumed to be normal, but these were not formally tested. Unless stated otherwise, all data are represented as means ± s.e.m.

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CHAPTER V

ROLE OF THE BNST IN AVERSIVE LEARNING AND MEMORY****

Introduction

The bed nucleus of the stria terminalis (BNST) is a diverse cluster of neuronal nuclei located within the ventral forebrain of humans and other animals (Dumont 2009). The connectivity of the bilateral BNST (or sometimes BST) is extensive and far-reaching—the BNST is interconnected with the amygdala, dorsal raphe, hippocampus, hypothalamus, medulla, nucleus accumbens, periaqueductal gray, prefrontal cortex, thalamus, ventral tegmental area, among others (for recent reviews, see Avery et al. 2016; Lebow and Chen 2016). As a result of this connectivity, it is perhaps not surprising that the BNST has been implicated in a number of functions and behaviors relevant to psychiatric disorders, including the acquisition and expression of Pavlovian fear conditioning, reinstatement of drug seeking, negative affect in pain, compulsivity, the expression of social defeat and learned helplessness, social attachment and reproductive behaviors, and regulation of the stress axis (Davis et al. 2010; Hammack et al. 2012; Crestani et al. 2013; Petrulis 2013; Adhikari 2014; Coria-Avila et al. 2014; Stamatakis et al. 2014; Takahashi 2014; Fox et al. 2015; Kash et al. 2015; Minami and Ide 2015; Avery et al. 2016; Daniel and Rainnie 2016; Gungor and Paré 2016; Lebow and Chen 2016; Mantsch et al. 2016; Waraczynski 2016; Laman-Maharg and Trainor 2017; Vranjkovic et al. 2017).

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Moreover, a growing body of research links BNST function (and its dysfunction) to a number of human pathological disorders such as anxiety and addiction (Fox et al. 2015; Avery et al. 2016; Lebow and Chen 2016)—disorders that are widespread, extremely costly to the individual, and often comorbid (Kessler et al. 2005a,b; Koob 2009; McEwen 2012; Whiteford et al. 2013; DiLuca and Olesen 2014; Gonzalez and Martinez 2014). Accordingly, the BNST represents an important target for therapeutic interventions aimed at treating various psychopathologies.

Within the realm of aversively motivated behaviors, early studies suggested a limited role of the BNST in fear conditioning to only certain stimulus modalities (e.g., LeDoux et al. 1988). It has been suggested that temporal factors (either in terms of the duration of the antecedent stimulus or consequent behavioral response) explain BNST's selective function in learned fear (e.g., Davis et al. 2010). Further, it is now understood that different populations of neurons within the BNST can bidirectionally regulate various unlearned anxiety-like responses (Jennings et al. 2013; Kim et al. 2013; Crowley et al. 2016; Marcinkiewcz et al. 2016; Mazzone et al. 2016). Despite this progress, we still lack an updated and integrated view of BNST function that accounts for its diverse contributions to aversive learning and memory. Accordingly, the purpose of this review is to dissect the current literature in an effort to provide a cohesive analysis of BNST function in Pavlovian fear conditioning and how this might relate to its roles in stress- and anxiety-like behaviors. While this review focuses primarily on animal studies, we also examine recent and relevant developments in human BNST research. We will begin by addressing the fundamentals of aversive learning, followed by a review of the BNST's relationship with other conditioned fear-regulating regions of the brain. In subsequent sections, we will address the role of the BNST in the conditioning and expression of fear in detail. Finally, we will consider how

these results may be unified under an updated model of conditioned fear-related BNST function. Based on a growing and converging data set, we argue that an overarching function of the BNST in humans and other animals is to generate defensive behaviors to unpredictable threats independent of their modality or duration.

Learning to fear

Pavlovian conditioning is the process through which animals learn associations between stimuli (Pavlov 1927). For aversive events, *Pavlovian fear conditioning*models how humans and other animals learn about threats in their environment (Rescorla 1988; LeDoux 2000; Maren 2001; Phelps and LeDoux 2005). Importantly, the conditioning, extinction, and relapse of fear may contribute to and interact with trauma-related psychopathologies such as post-traumatic stress disorder (PTSD) (Jovanovic and Ressler 2010; Mahan and Ressler 2012; Milad and Quirk 2012; Goswami et al. 2013; Gonzalez and Martinez 2014; VanElzakker et al. 2014; Careaga et al. 2016; also, see LeDoux 2012, 2014, 2017; LeDoux and Pine 2016; LeDoux and Brown 2017). In specific terms, Pavlovian fear conditioning is a process through which a salient cue (e.g., a tone or light source) is paired with an unavoidable and noxious outcome (e.g., electric shock). Exposure to the shock (the *unconditioned stimulus*, or US) induces various species-specific "circa-strike" defensive responses (termed *unconditioned responses*) (e.g., escape, defensive fighting, etc.; Bolles 1970; Bolles and Fanselow 1980; Fanselow 1980, 1994). Through the process of conditioning, the cue comes to predict the aversive outcome (hence, termed the *conditioned stimulus*, or CS), and with one or more pairings with the US, a "post-encounter" *conditioned response* (e.g., freezing and autonomic activity in rodents) to the CS alone emerges. In addition to freezing in the presence of a shock-paired CS, animals will

suppress instrumental responses for food (a phenomenon termed *conditioned suppression*; e.g., Waddell et al. 2006, 2008) and will increase the magnitude of their startle responses to other loud acoustic stimuli (termed *fear-potentiated startle*; e.g., Lee and Davis 1997). In humans, conditioned fear is often indexed using physiological measures, including skin conductance, heart rate, and pupil dilation (Lonsdorf et al. 2017). Fear conditioning can occur in the absence of a discrete CS (the US is "unsignaled"); in this case, the environment or "context" serves as the CS (and is referred to as *contextual conditioning*; Rudy et al. 2004; Curzon et al. 2009; Maren et al. 2013; Urcelay and Miller 2014). Standard conditioning procedures to a discrete CS often result in at least some concurrent contextual conditioning as the discrete CS may not fully acquire all of the associative strength of the US (Rescorla and Wagner 1972).

In contrast to conditioning, repeated presentations of the CS in the absence of the US will ultimately lead to a reduction in conditional responding, a process termed *extinction* (Pavlov 1927; Myers and Davis 2002; Chang et al. 2009). Numerous studies indicate that extinction results in a new inhibitory memory that suppresses conditional fear in a context-dependent manner (Maren 2011). Specifically, fear to an extinguished CS will return when that CS is presented outside of the extinction context, a fundamental form of "relapse" termed *renewal* (Bouton and Bolles 1979a). Renewal is not the only way in which fear can relapse: fear *reinstates* after reexposure to the US (Rescorla and Heth 1975; Bouton and Bolles 1979b; Bouton and King 1983; Westbrook et al. 2002; Morris et al. 2005; Goode et al. 2015a) and fear can *spontaneously recover* after a passage of time in the absence of the CS (Pavlov 1927; Rescorla 2004). Distinct mechanisms are thought to underlie these and other various forms of relapse (and are examined elsewhere in detail: Bouton 2002, 2004; Vervliet et al. 2013; Goode and Maren 2014; Haaker et al. 2014; McConnell and Miller 2014; Maren and Holmes 2016), but

it should be noted that contextual information is thought to be critical for many of these phenomena (Bouton et al. 2006).

Neural circuits for aversive learning and memory

Originally considered a subregion of the "extended amygdala" (Johnson 1923; Alheid and Heimer 1988; Alheid et al. 1998; Alheid 2003), the BNST has numerous direct connections with other areas of the brain that are involved in Pavlovian fear conditioning, including the amygdala, hippocampus, and prefrontal cortex (PFC). Brain circuits for the acquisition and expression of conditioned fear as well as for its extinction and relapse have received considerable attention over the years (Fendt and Fanselow 1999; LeDoux 2000; Maren 2001; Maren and Quirk 2004; Quirk and Mueller 2008; Herry et al. 2010; Orsini and Maren 2012; Furini et al. 2014; Izquierdo et al. 2016). In brief, CS and US signals converge on the lateral nucleus (LA) of the amygdala and plasticity within this nucleus is vital for the acquisition, consolidation, and expression of conditioned fear (Rogan et al. 1997; Maren 1999a, 2005; Johansen et al. 2011). Output from the amygdala, via the central nucleus of the amygdala (CeA), targets downstream structures such as the periaqueductal gray (PAG) and hypothalamus to engage freezing and stress responses (respectively) in the presence of conditioned cues (LeDoux et al. 1988; Behbehani 1995; McLemore et al. 1999; Keifer et al. 2015; Tovote et al. 2015). Additionally, the hippocampus—by way of its connections with the PFC and amygdala—fundamentally regulates the acquisition and expression of contextual fear in a time-dependent manner (Kim and Fanselow 1992; Phillips and LeDoux 1992; Maren et al. 1998, 2013; Fanselow 2000; Fanselow and Dong 2010; Xu et al. 2016). Furthermore, PFC has been shown to drive or impair extinction via its projections to fear-promoting or -inhibiting neurons within the amygdala (Vertes 2004; Quirk et

al. 2006; Hoover and Vertes 2007; Herry et al. 2008; Knapska et al. 2012; Senn et al. 2014; Adhikari et al. 2015; Rozeske et al. 2015; Giustino and Maren 2015; Gourley and Taylor 2016)—processes that are regulated by the hippocampus (Ji and Maren 2007, 2015a,b; Goosens 2011; Maren et al. 2013; Orsini et al. 2011; Xu et al. 2016).

The BNST is well positioned to integrate information from the amygdala, hippocampus, and PFC (Weller and Smith 1982; Sun et al. 1991; Canteras and Swanson 1992; McDonald et al. 1999; Dong et al. 2001a; Reynolds and Zahm 2005; Jalabert et al. 2009; deCampo and Fudge 2013; Torrisi et al. 2015; Lebow and Chen 2016; Oler et al. 2017; Reichard et al. 2017), and BNST subregions may have differential roles in this process (for recent reviews, see Lebow and Chen 2016; Gungor and Paré 2016). Nevertheless, the functions of these circuits in fear conditioning are not well characterized. BLA activity appears to be required for BNST-dependent fear behaviors in most cases, insofar as BLA lesions block both phasic and long-lasting fear responses even with the BNST intact (Maren et al. 1996; Maren 1999b; Davis et al. 2010; but, see overtraining studies: Poulos et al. 2010; Zimmerman and Maren 2011). However, it is not yet clear if neurons required for BNST-dependent or -independent conditioned fears are distinct or overlapping within the BLA (Davis et al. 2010). Furthermore, it is unclear if direct projections from the BLA are required for BNST-dependent aversive learning and memory, particularly because photostimulation of these afferents produces nonassociative anxiolytic effects (Kim et al. 2013; Crowley et al. 2016).

The CeA also densely innervates the BNST, but the role of the CeA in BNST-dependent defensive behaviors has been an area of debate. There is evidence that these structures mediate different aspects of conditioned fear (Walker and Davis 2008; Walker et al. 2009; Davis et al. 2010), although others have suggested that their roles in these processes are similar (Fox et al.

2015; Gungor and Paré 2016; Shackman and Fox 2016, also, see Gorka et al. 2017). That said, there are some recent and compelling data indicating that the CeA is required for BNST-dependent conditioned fears. For example, Asok and colleagues (2017)demonstrated that optogenetic silencing of central amygdala CRF-positive afferents in the BNST during training blunts fear expression to a shock-associated context, at least in the later portion of the retrieval (note that it is possible that other circuits may be involved and at different stages). The anxiogenic functions of the BNST are generally attributed to its anterior regions, (see Crown et al. 2000; Kocho-Schellenberg et al. 2014) a region targeted by CeA (and BLA) neurons (Gungor and Paré 2016).

Beyond the amygdala, the significance of hippocampal inputs to the BNST in the context of aversive learning is not well understood. The hippocampus exerts inhibitory control over stress hormone release (via the hypothalamic–pituitary–adrenal [HPA] axis) through its glutamatergic projections to the BNST (Cullinan et al. 1993; Forray and Gysling 2004). Thus, projections from the hippocampus to the BNST may modulate anxiety (and perhaps BNST-dependent fear) not by driving defensive responses per se but by reducing stress responses in particular contexts (Glangetas et al. 2017; also, see Gorka et al. 2017). The PFC, particularly the infralimbic (IL) region of the PFC, projects strongly to the BNST—this circuit (along with BNST-projecting cells from the neighboring orbitofrontal cortex) may be involved in both reward (Jalabert et al. 2009; Reisiger et al. 2014) and threat processing (Spencer et al. 2005; Fox et al. 2010; Motzkin et al. 2015). Nonetheless, a role for IL projections to the BNST in conditioned fear has not been explored. The prelimbic (PL) region of the PFC has been shown to play important roles in contextual conditioning (e.g., Corcoran and Quirk 2007; Ye et al. 2017), but its direct projections to the BNST are sparse. Outside of these circuits, recent work on

serotonergic inputs to the BNST has implicated dorsal raphe afferents in enhanced fear conditioning (Marcinkiewcz et al. 2016).

BNST efferents extensively target the CeA, but moderately to sparsely terminate in the PFC, BLA, and hippocampus (Dong et al. 2000, 2001b, Dong and Swanson 2003, 2004a,b, 2006a,b,c; Gungor et al. 2015; Krüger et al. 2015; Dabrowska et al. 2016; Kaufling et al. 2017; Oler et al. 2017); little is known regarding the roles of these circuits in aversive memories. BNST efferents are largely GABAergic, with a smaller portion consisting of glutamatergic neurons (Tovote et al. 2015; Vranjkovic et al. 2017; also, see McElligott et al. 2013; Avery et al. 2014; Kaufling et al. 2017). BNST subregions are highly interconnected (Turesson et al. 2013), suggesting that BNST-dependent behavioral responses reflect an integration of activity within these areas (Kim et al. 2013; Gungor and Paré 2016). Outside of its connections with the amygdala, PFC, and hippocampus, the BNST is positioned to elicit defensive behavior via direct projections to the hypothalamus and PAG (Holstege et al. 1985; Gray and Magnuson 1992; Nagy and Paré 2008). Finally, it is worth noting that in humans (Allen and Gorski 1990; Chung et al. 2002) and rodents (Hines et al. 1985; Hines et al. 1992), the male BNST is generally larger than in females (also, see Avery et al. 2014). It is not yet clear if this sexual dimorphism impacts BNST function in aversive learning, but (perhaps relatedly) male rodents generally express greater levels of contextual (but not discretely cued) freezing when compared with females (Maren et al. 1994; Markus and Zecevic 1997; Pryce et al. 1999; Gupta et al. 2001; Barker and Galea 2010; Nagaya et al. 2015; Acca et al. 2017; Bangasser and Wicks 2017; also, see Gruene et al. 2015; Pellman et al. 2017). With these connections in mind, we will now explore the various factors that may account for the roles of the BNST in conditioned fear.

BNST function in response to unconditioned aversive stimuli

Exposure of animals to aversive events—including both physical (e.g., unsignaled footshock, restraint) and psychological stressors (e.g., open or elevated spaces, bright lights, predator odors, alarm pheromones)—readily engage or influence signaling within the BNST (Rosen et al. 2015; Daniel and Rainnie 2016; Gungor and Paré 2016). Currently, it is understood that BNST neurons do not react uniformly to these various stressful stimuli. For example, the BNST has been shown to exhibit alterations (albeit, increases or decreases depending on the study) in immediate early gene expression in its anterolateral and anteroventral regions after restraint alone, inescapable tailshock, or predator odor (Lino-de-Oliveira et al. 2001; Day et al. 2005; Christianson et al. 2011; Butler et al. 2016). Electrophysiological studies have further shown that aversive footshock exposure can rapidly recruit and modify activity in BNST neurons (Marcinkiewcz et al. 2016; also, see Daldrup et al. 2016). In turn, BNST lesions often reduce or eliminate the behavioral and physiological changes (termed *unconditioned fear* responses) that come with direct exposure to these aversive stimuli. For example, BNST lesions block freezing responses in the presence of predator odors (Fendt et al. 2003, 2005). Additionally, stress (in the form of extensive footshock exposure) can potentiate acoustic startle in a separate context; lesions of the BNST block this effect (Gewirtz et al. 1998; also, see Hammack et al. 2004; Meloni et al. 2006). In cases where BNST lesions fail to alter unconditioned stress responses (e.g., Treit et al. 1998), it is thought that this may be due to the disruption of both stress-promoting and -attenuating circuits within the BNST (Adhikari 2014; Luyck and Luyten 2015). Nevertheless, the BNST functions, in part, to generate unconditioned stress responses and to mediate stress-induced sensitization.

Along these lines, BNST manipulations can also *induce* unconditioned stress and fear- or anxiety-like responses in a subregion-specific and neurotransmitter system-dependent manner (Levita et al. 2004; Hammack et al. 2009b; Daniel and Rainnie 2016). For example, increasing CRF, calcitonin gene-related peptide (CGRP), or serotonin signaling within the BNST can potentiate acoustic startle in the absence of any other training, and tends to increase anxiety in other tasks in the short term (Lee and Davis 1997; Sahuque et al. 2006; Lee et al. 2008; Sink et al. 2011, 2013b; Mazzone et al. 2016). Similarly, β-adrenergic agonism in the BNST or induction of pituitary adenylate cyclase-activating polypeptide (PACAP) signaling within the BNST promotes stress and anxiety-like responses (Deyama et al. 2008; Hammack et al. 2009a, 2010; Naka et al. 2013; Hammack and May 2015). Increasing nitric oxide production within the BNST has also been shown to induce unconditioned freezing in a novel arena (Faria et al. 2016; also, see Deyama et al. 2017). Furthermore, stimulation or inhibition of select BNST circuits, including BLA→BNST and BNST→VTA neurons, can increase or decrease avoidance (or modulate stress responding) without any prior learning (Jennings et al. 2013; Kim et al. 2013; Crowley et al. 2016; Marcinkiewcz et al. 2016; Mazzone et al. 2016).

Stress may lead to plasticity in the BNST that will ultimately affect circuit function during future stressors or tasks. For example, acute restraint stress significantly alters plasticity in the BNST in response to PFC-dependent input (Glangetas et al. 2013). Chronic stress in the form of multiday unpredictable shock exposure generally increases serotonin release in the BNST and alters serotonin receptor expression in the BNST (Hazra et al. 2012). Additionally, it has been shown that stress-enhancement of trace eyeblink conditioning in rats (through the use of restraint and tail shock) is mediated by the BNST (Bangasser et al. 2005; Bangasser and Shors 2008). From a translational perspective, and in light of pathologies in which patients may have

experienced a significant degree of stress, these data are important to consider when examining unconditioned anxiety- and (perhaps) conditioned fear-related function in the BNST. Indeed, circuit-specific manipulations often occur in animals where stress history is minimal (Belzung et al. 2014). As such, important questions remain as to whether the effects seen in the circuit-selective studies (Jennings et al. 2013; Kim et al. 2013; Crowley et al. 2016; Marcinkiewcz et al. 2016; Mazzone et al. 2016) remain true following a history of stress and whether plasticity in the BNST shifts the phenotypic function of any of these circuits (also, see Conrad et al. 2011). In total, the BNST processes unconditioned aversive stimuli, but it is important to consider that negative outcomes may occur in a distinct place and in the presence of particular cues, which may foster associative learning.

BNST function in fear conditioning: stimulus modality and duration

BNST lesions (whether permanent or temporary) do not universally blunt somatic, autonomic, or hormonal responses during fear conditioning. Rather, several studies have now demonstrated a necessary role for the BNST in the learning and/or expression of *contextual*—but not discretely *cued*—fear, as indexed by freezing, conditioned suppression, potentiated startle, and stress hormone release (LeDoux et al. 1988; Hitchcock and Davis 1991; Lee and Davis 1997; Gewirtz et al. 1998; Sullivan et al. 2004; Waddell et al. 2006; Resstel et al. 2008; Duvarci et al. 2009; Poulos et al. 2010; Zimmerman and Maren 2011; Hott et al. 2012, 2017; Sink et al. 2013a; Davis and Walker 2014; Goode et al. 2015b; Hammack et al. 2015; Asok et al. 2016). Relatedly, electrical stimulation of the BNST can either increase or decrease conditioned contextual fear (as assessed by freezing or startle amplitude), effects that depend on the location, intensity, and frequency of the stimulation (Luyck et al. 2017; also, see Baas et al. 2014; Luyck

and Luyten 2015). Disrupting BNST signaling does not appear to impair discrimination between two nonaversive contexts per se (e.g., given the persistence of context-dependent renewal in BNST-lesioned animals in the study by Goode et al. 2015b), suggesting that contextual representations (e.g., spatial/visual properties, etc.) are processed upstream of the BNST in the hippocampus. It has not yet been demonstrated whether unconditional fear- and stress-attenuating circuits of the BNST (Jennings et al. 2013; Kim et al. 2013; Crowley et al. 2016; Marcinkiewcz et al. 2016; Mazzone et al. 2016) (or BNST neurons in general) play any fundamental role in the extinction of conditioned fear to cues or contexts (also, see Ranjan et al. 2017).

Some of the aforementioned studies involved pretraining permanent lesions of the BNST, making it difficult to determine whether the BNST's role in context fear is specific to acquisition, consolidation, expression, or some combination of these processes (granted, there are few studies published that specifically examine the role of the BNST in the acquisition or consolidation of fear). However, there are a handful of studies using temporary or post-training lesions (or inhibitors of protein synthesis) that implicate BNST function in the acquisition (Davis and Walker 2014; also, see Asok et al. 2017), consolidation (Poulos et al. 2010), and expression of context fear (Sullivan et al. 2004; Zimmerman and Maren 2011; Goode et al. 2015b; but, see Davis and Walker 2014). Consistent with these ideas, cued or contextual conditioning increases immediate early gene expression (e.g., c-fos) in the BNST (Passerin et al. 2000; Ranjan et al. 2017), as does the expression of contextual fear (Beck and Fibiger 1995; also, see Luyten et al. 2012). Furthermore, the BNST has been shown to be important for consolidation of contextual fear in overtrained animals if the BLA is lesioned (this consolidation effect is eliminated if the BLA remains intact; Poulos et al. 2010; Zimmerman and Maren 2011). These

effects on acquisition and consolidation suggest that BNST afferents (e.g., Asok et al. 2017) or perhaps BNST neurons themselves are at least *in part* a node for BNST-dependent fear memory in certain cases. However, overtraining studies suggest that the BNST is not an alternative locus for standard fear conditioning (Poulos et al. 2010; Zimmerman and Maren 2011). Thus, it is not yet clear whether plasticity within the BNST serves to store BNST-dependent conditioned fear memories and/or if the BNST is simply recruited by learning-dependent plasticity in other regions in the presence of particular conditioned stimuli. Collectively, these findings suggest a unique role for the BNST in contextual fear conditioning, but why the BNST is selective for contextual fear is unclear.

Conditioned contexts and discrete CSs not only differ in terms of their modality, but they also often differ in duration. To determine which factor is more relevant to BNST function, Hammack et al. (2015) tested whether the duration of context exposure prior to US onset in a context conditioning procedure influenced the role of the BNST in the task. Specifically, Hammack et al. (2015) placed rats in a context where unsignaled footshock occurred either 1 or 10 min after animals entered the chamber. Rats were removed from the chambers 30 sec after shock offset (thereby, the groups differed on both the timing of shock onset as well as total context exposure). After several training sessions, rats were tested in the absence of shock to the context. The results revealed that contextual fear was only affected by the BNST lesions in the context in which shock occurred at a 10-min delay; rats with BNST lesions conditioned normally to the context in which shock occurred at a 1-min delay. Importantly, these data suggest that contextual fear can be independent of the BNST under some conditions (which may also have interesting implications for context fear-induced reinstatement). Consistent with these findings, an earlier report by Waddell et al. (2006) demonstrated that

lesions of the BNST attenuated conditioned suppression in the presence of a long-duration (10 min), but not a short-duration (1 min), auditory CS. Based on these results, the authors (Waddell et al. 2006; Hammack et al. 2015) argued that it was stimulus duration, not modality or response duration, that determined whether the BNST was recruited during fear conditioning procedures. However, stimulus duration alone may not fully account for the recruitment of the BNST during fear conditioning. For example, BNST lesions prevent fear reinstatement to short-duration CSs (Waddell et al. 2006, 2008; Goode et al. 2015b). Likewise, shock-induced reinstatement of extinguished fear to a discrete CS is associated with increased activity in the human BNST (Scharfenort and Lonsdorf 2016). Furthermore, BNST lesions can enhance discrimination between a CS+ and CS− (Duvarci et al. 2009; Radke 2009) by attenuating fear to the CS− (also, see Botta et al. 2015; De Bundel et al. 2016; Sanford et al. 2017). Thus, the BNST may also be involved in the generalization of conditioned fear to both discrete cues and contexts (also, see Jasnow et al. 2017). Similarly, serotonin in the BNST during training to a phasic CS has been shown to increase fear responding to that same CS when tested off-drug in a familiar but different context (Ravinder et al. 2013; however, it is unclear if these effects are confounded by enhanced contextual fear on top of the tone response at test; also, see Marcinkiewcz et al. 2016). In total, there are many circumstances in which the BNST regulates fear to unimodal or even discrete stimuli.

BNST function in fear conditioning: response duration

Early and seminal research on the role of downstream targets of the BLA in aversive learning demonstrated a double dissociation in the roles of the BNST and CeA in sustained and phasic fear responses, respectively (Lee and Davis 1997; Walker and Davis 1997; but,

see Sullivan et al. 2004). In particular, CRF- and unconditioned light-enhanced startle—paradigms associated with long-duration fear-like responses—were shown to be mediated by the BNST (and not the CeA); conversely, fear-potentiated startle, which involves a phasic CS-evoked fear response, was attenuated by CeA lesions (and not the BNST) (Lee and Davis 1997). In this framework, the BNST was argued to be necessary to maintain long-lasting fear responses, whereas the CeA drives rapid, phasic fear responses (Davis 1998, 2006; Davis and Shi 1999; Walker et al. 2009; Davis et al. 2010; Rodríguez-Sierra et al. 2016; also, see Herrmann et al. 2016; Brinkmann et al. 2017a).

Nevertheless, a growing body of evidence indicates that the BNST mediates both rapid and sustained fear responses at least in some cases (also, see Nagy and Paré 2008). For example, work in humans has revealed that the BNST can exhibit rapid and short-lived neural responses to phasic images of an approaching tarantula or to relatively brief unpredictable threats of shock (Mobbs et al. 2010; Choi et al. 2012; Klumpers et al. 2015; Shackman and Fox 2016; also, see Schlund et al. 2013). At the behavioral level, post-training lesions or inactivation of the BNST rapidly attenuate freezing responses to an aversive context (e.g., as early as within the first minute; Zimmerman and Maren 2011; Goode et al. 2015b)—these effects coincide with rapid prevention of reinstatement to the onset of discrete extinguished CSs. Other studies examining the effects of various neuromodulators or neurosteroids within the BNST have also shown rapid alterations in behavioral responding upon return to a conditioned context (Nagaya et al. 2015; Acca et al. 2017). At the physiological level, Resstel et al. (2008) demonstrated that blockade of neurotransmitter release within the BNST (via the infusion of cobalt chloride) prevented the immediate increase in mean arterial pressure and heart rate that coincided with being placed in a previously conditioned context. Intra-BNST administration of NMDA

antagonists or nNOS inhibitors also blocks these rapid physiological changes (Hott et al. 2017). These data suggest that the BNST does not selectively mediate sustained fear responses.

BNST function in fear conditioning: state-dependence

Recently, it has been observed that intra-BNST infusions of the neurosteroid allopregnanolone (ALLO, a progesterone metabolite that potentiates $GABA_A$receptors) produce state-dependent retention deficits of contextual fear (Nagaya et al. 2015; Acca et al. 2017). In other words, animals trained *or* tested after ALLO infusions exhibit impaired contextual freezing, however animals trained *and* tested after ALLO infusions exhibit robust freezing. This suggests that the BNST not only processes environmental (i.e., exteroceptive) conditioned contexts, but might also be involved in representing interoceptive contexts (such as hormonal states). Moreover, state-dependence is not observed when ALLO is infused into the BLA, suggesting that the effects of ALLO on state-dependence relates to its actions within the BNST (Acca et al. 2017). However, it is not yet clear if other drugs that are commonly used to assess BNST function also induce state-dependence via the BNST, or if other brain areas might mediate these state-dependent effects. For example, infusions of NBQX (an AMPA receptor antagonist) or muscimol (a GABA receptor agonist) into the BNST did not cause renewal of fear to an extinguished CS as might be expected if there was a drug-induced shift in the animals interoceptive context (i.e., interoceptive renewal; Goode et al. 2015b). Nevertheless, when examining the role of the BNST in conditioned fear, it is important to consider the role of interoceptive contexts that may be associated with the aversive event; a change in interoceptive context might induce state-dependent generalization decrements.

Temporal unpredictability in BNST-dependent aversive learning and memory

Up to this point, we have reviewed studies that suggest that the BNST (1) is particularly attuned to aversive (US-like) stimuli, (2) is implicated in acquisition, expression, reinstatement, and at times consolidation of conditioned fear, (3) *does not* mediate all forms of contextual fear, (4) mediates fear to unimodal or multimodal stimuli, (5) can respond to phasic or sustained cues, (6) can exhibit phasic or sustained neural responses in the presence of threats, (7) may be involved in aversive learning to interoceptive states, and (8) can rapidly mediate defensive behaviors. What unifies these properties and what may account for BNST's selectivity in fear conditioning? We propose that the BNST is specifically recruited to aversive learning by *temporally unpredictable events* (Fig. 23).

By this view, the BNST is not involved in aversive *contextual* conditioning or expression per se, rather it becomes engaged by stimuli (whether cues or exteroceptive/interoceptive contexts) that are associated with *temporally*unpredictable USs (even if the probability that the US will occur is 100%). In other words, the BNST is recruited when the animal cannot reliably predict the onset of shock. This account of BNST function explains its diverse roles in conditioning to stimuli of various modalities or durations. For example, the BNST mediates fear to long CSs (whether unimodal or multimodal) because long CSs are poor predictors of when the aversive US will occur during presentation of the stimulus (e.g., Waddell et al. 2006; Hammack et al. 2015; also, see Fig. 1E,G). Conversely, discrete CSs (whether contexts or cues) that are trained with near immediate shock (Fig. 1A,B) allow the animal to reliably predict US onset and thereby do not require the BNST. However, the BNST is required for conditioning to relatively short, unimodal CSs if those CSs are trained as poor predictors of when a US occurs (Fig. 1C; Lange et al. 2016).

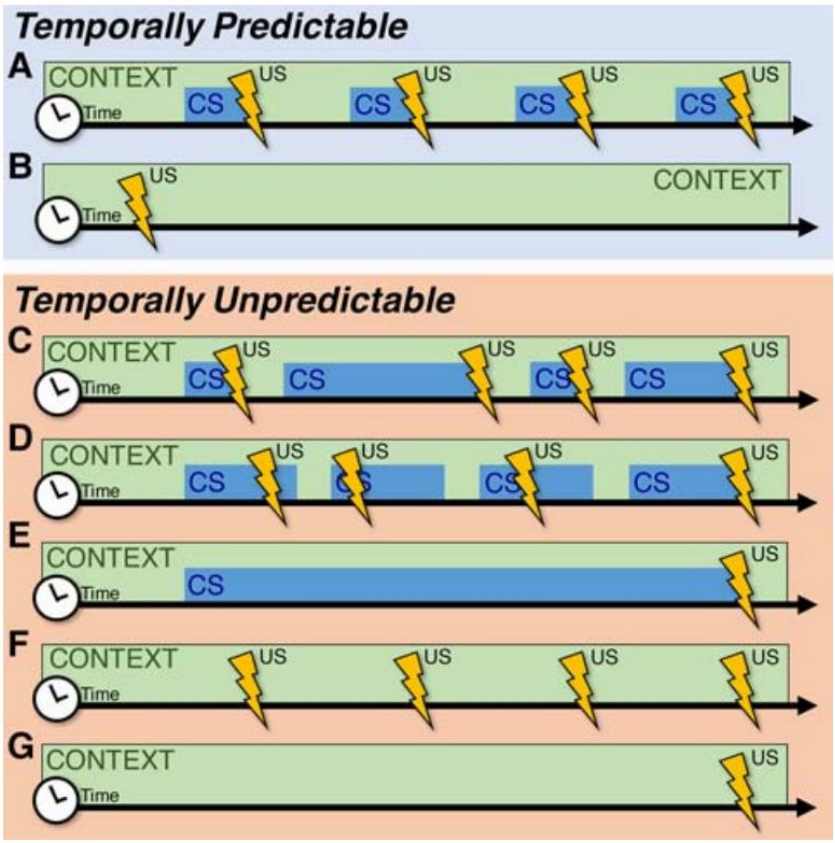

Figure 23. Temporally predictable and unpredictable aversive conditioning procedures. Standard fear conditioning procedures produce temporally predictive discrete CSs that do not require the BNST—fear to the conditioning context may be BNST-dependent given that the context is a poor predictor of shock onset **(A)**. Contextual conditioning with early—but not necessarily immediate—shock onset, however, is temporally predictive of the US, and may therefore be BNST-independent (this procedure may require multiple training sessions and may not necessarily require extensive context exposure post-shock) **(B)**. Temporally unpredictable conditioned stimuli can be generated by varying the duration of the CS across conditioning trials **(C)**, randomizing the onset of shock during presentation of a CS **(D)**, extending the duration of the CS to exhibit remote shock onset **(E)**, or conditioning a context with multiple unsignaled and temporally unpredictive shocks **(F)** or late shock onset **(G)**. BNST circuitry has been implicated in all of these cases of temporally unpredictable aversive stimuli (outside of example D, which has not yet been tested).

This interpretation of BNST function is perhaps specific to its role in aversive learning—that is, temporal uncertainty of a US may foster BNST-dependence to various CSs, whereas nonassociative stressors (serving as USs) may engage the BNST for reasons not necessarily related to timing. Nevertheless, time as a factor in unconditioned stress is plausible (e.g., bright

lights may signal a degree of vulnerability during which the animal is uncertain of the time in which a direct threat or predator will appear), but such possibilities are still in need of exploration.

One possibility is that unpredictable threats operate to produce sustained fear as the animal has learned that the risk of US onset is nearly continuous throughout presentation of the CS—these sustained fear responses have been argued to require the BNST (e.g., Walker and Davis 2008; Walker et al. 2009; Davis et al. 2010). However, temporally predictable CSs (albeit, massed) or contexts (e.g., Hammack et al. 2015) can also produce long-lasting and sustained fear responses, such as freezing behaviors, that do not require the BNST (e.g., Zimmerman and Maren 2011). Hence, it is possible that neither the duration of the fear response nor the duration of the CS is the determinant of when or whether the BNST is recruited to mediate conditioned fear responses.

Of course, there is considerable variability in animals and individuals in terms of how accurately they time the onset of aversive events (also, see Buhusi and Meck 2005). Thus, the role of the BNST in temporal predictability may need to be addressed by comparing responses to temporally predictable (Fig. 1A,B) and unpredictable antecedents of aversive outcomes (Fig. 1C–E). The number of studies utilizing temporally uncertain discrete CSs are limited (e.g., Daldrup et al. 2015; Lange et al. 2016; Seidenbecher et al. 2016), but they often train the CS with components of both immediate and delayed US onset (thereby contributing to its temporal uncertainty). Fear to these stimuli is then tested to a continuous presentation of the CS over the course of several minutes. Only the late phases of CS presentation appear to require the BNST (Davis et al. 2010; also, see Meloni et al. 2006). Accordingly, we argue that in these cases these early phases of retrieval are akin to temporally predictable CSs, whereas the later times of

CS exposure are temporally unpredictable of US onset. By training the CS with early shock onset (as well as late onset), the animals have learned that CS onset could possibly predict immediate shock—only after sustaining the CS does the uncertainty arise regarding when the US might occur. Along these lines, if the CS is paired with temporally certain shock (i.e., early shock onset), its retrieval is BNST-independent and does not elicit sustained responding. Thus, we propose that temporal uncertainty, which may produce sustained fear, accounts for the BNST's diverse contributions to aversive learning and memory. Note that other forms of unpredictability—such as CS–US contingency (e.g., Davies and Craske 2015)—might also interact with temporal unpredictability (also, see Alvarez et al. 2011; Robinson et al. 2012; Schmitz and Grillon 2012).

It is not yet clear if the conditioning of temporally uncertain stimuli relies on plasticity within and/or upstream of the BNST, but recent studies comparing predictable and unpredictable threats have implicated the amygdala (e.g., Herry et al. 2007), amygdalar afferents to the BNST, and activity/endocannabinoid signaling within the BNST itself in the response to temporally unpredictable threats (Davis et al. 2010; Lange et al. 2016). Additionally, pharmacological or optogenetic inhibition of the dorsal hippocampus has been shown to attenuate fear to temporally unpredictable (but not predictable) auditory CSs (e.g., Fig. 1D; Amadi et al. 2017)—manipulations that also disrupt contextual fear.

In total, we propose that the BNST mediates learned fear when the *timing* of an aversive event is uncertain, even in the face of certainty that the event will happen. Indeed, this interpretation is consistent with other recent accounts of BNST broader functions. For example, the BNST has been proposed to be involved in “valence surveillance” (Lebow and Chen 2016), which includes monitoring positive and negative stimuli and initiating appropriate behavioral

and physiological reactions. Unpredictable stressors (such as temporally unpredictable CSs) may require ongoing monitoring via the BNST—such hypervigilance to threat of shock has been associated with activity in the BNST in anxious humans (Somerville et al. 2010). Ultimately, the role of the BNST in mediating fear responses to temporally unpredictable threats is likely an important factor in the role of the BNST in human anxiety, given that ambiguity is thought to be a core component of anxiety (Foa et al. 1992; Bouton et al. 2001; Grillon 2002a,b, 2008; Perusini and Fanselow 2015). Notably, there have been several recent advances in imaging techniques of the human BNST, which will help to better characterize the role of the BNST in aversive learning and in clinical psychopathologies (Fox et al. 2015; Torrisi et al. 2015; Avery et al. 2016; Brinkmann et al. 2017a,b; Pedersen et al. 2017; Sladky et al. 2017; Theiss et al. 2017). On a final note, an emphasis on temporal uncertainty might have implications for BNST's additional roles in drug seeking behaviors (Shaham et al. 2003; Flavin and Winder 2013; Silberman and Winder 2013), given that footshock exposure can induce both fear and drug reinstatement (e.g., Erb and Stewart 1999; Erb et al. 2001; Shalev et al. 2001). All of this considered, future experiments will hopefully shed light on the precise circumstances and circuits by which conditioned and unconditioned stimuli engage the BNST.

General conclusions and implications for the dissertation

In the current dissertation, we have rigorously examined the behavioral and brain systems that contribute to fear in uncertain circumstances, such as during relapse and in the aftermath of conditioning to cues that may not reliable signal the onset of an aversive stimulus. While the implications of these data and analyses are examined in detail in the prior sections and chapters, there are several important consequences that are worth highlighting and discussing as we

conclude. From Chapter II, we show that relapse can be both robust and long lasting (long after the offset of the aversive trigger). Thus, these data highlight the risk and persistence of relapse and suggest that its mitigation may require considertation of time points long after the potential relapse-inducing stressor. Furthermore, we show that shock-associated cues (in this case, an aversive shock-associated context) can themselves induce relapse—these data demonstrate the risk of psychological stressors (rather than direct exposure to a physical stressor) in disrupting retention of extinguished fear. Interestingly, we show that places that have solely hosted extinction training may help to reduce the persistence of relapse, suggesting that relapse-prone individuals might benefit from seeking out safe enviroments during relapse. From Chapter III, we have shown that BNST is selectively involved in the reinstatement (but not the renewal) of fear. Accordingly, the BNST appears to regulate forms of relapse that depend on recent stress and threat uncertainty. Importantly, these data suggest that there are indeed distinct mechanisms of relapse that may depend on different brain structures. Furthermore, these data indicate that brain measures to prevent relapse may not always be effective for relapse across its various forms. In Chapter IV, we have demonstrated that the recruitment of the BNST to fear expression (and thereby, relapse) is dependent on threats that do not reliable signal the onset of an aversive event. These effects appear independent of the length and modality of the cue, and do not seem to depend on the magnitude of the aversive shock *per se*. Additionally, we found that the response itself, whether long-lasting or shorter in duration, did not seem to predict BNST's involvement. Interestingly, we observed activity (as measured by Fos) in ventral structures of the BNST in response to the uncertain threat, suggesting that there may be some subregion-specific roles of the BNST in fear regulation. Furthermore, our work suggests that the BNST may coordinate information from the basolateral amygdala and hippocampus during fear expression

more broadly, but that BNST-targeting afferents from the infralimbic cortex may also play a selective role fear to uncertain threats. Of the data sets included in this dissertation, the work in Chapter IV may have the most important implications for clinical anxiety disorders. That is, in everday life, we are bombarded by information that may or may not precede or follow aversive events. Thus, it is important to consider how the brain may learn about actual as well as potential threats—and, how these learning processes may contribute to anxiety when animals don't know precisely when a negative event will occur (but nevertheless expect it). Of course, and as discussed in previous chapters, a major feature of anxiety is the uncertainty that patients experience when they ruminate on negative outcomes but without certainty of when they will occur. Perhaps these features involve coordination of information from the aforementioned afferents with the BNST. To conclude, the data contained in this dissertation provide novel insights into mechanisms of relapse and BNST-dependent aversive learning and memory. At large, our understanding of the BNST is only just beginning—it is my sincere hope that these data will help to push forward insights into its function.

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